Impact of an alternative ageing technology using micro-oxygenation on the wine spirit's antioxidant activity

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To my family

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Anna Nocera

Abstract

The study of antioxidant activity, a quality parameter, was performed during the first year of a *Lourinhã* wine spirit's ageing, through two different ageing technologies: traditional technology using 250 L barrels, and alternative technology using 1000 L stainless steel tanks with wooden staves and micro-oxygenation. In both technologies, the same types of wood were used: Limousin oak and chestnut, and mixture of them, with medium plus toasting level. The quantity of the wooden staves added to the stainless steel tanks was calculated to reproduce the surface area to volume ratio of 250 L wooden barrel, therefore, to be comparable. The tanks were provided with micro-oxygenation during the ageing period, simulating the amount of oxygen entering through the barrels.

The alternative technology induced higher antioxidant activity along with faster enrichment in wood derived phenolic compounds. In addition, the antioxidant activity, the total phenolic content and the low molecular weight phenolic compounds concentrations determined by HPLC exhibited significant positive correlations. The results also showed that alternative technology can be used as a valid method of ageing wine spirits since it allowed obtaining a high quality aged beverage through a more economically and environmentally sustainable process. A synergistic effect with the chestnut wood was also observed.

Keywords

Wine spirit – antioxidant activity – DPPH – ageing technology – micro-oxygenation

Resumo

O estudo da atividade antioxidante, um parâmetro da qualidade, foi realizado durante o primeiro ano de envelhecimento de aguardente vinica *Lourinhã*, através de duas diferentes tecnologias de envelhecimento: tecnologia tradicional utilizando barris de 250 L e tecnologia alternativa utilizando tanques de aço inoxidável de 1000 L com aduelas de madeira e micro-oxigenação. Em ambas as tecnologias foram utilizados os mesmos tipos de madeira: carvalho Limousin e castanheiro, e mistura de ambas, com queima média mais. A quantidade de aduelas inserida nos tanques de aço inoxidável foi calculada para reproduzir a relação superfície / volume de um barril de 250 L, para ser comparável. Foi aplicada micro- oxigenação nos tanques durante o período de envelhecimento, simulando a quantidade de oxigênio que entra nos barris.

A tecnologia alternativa induziu uma maior atividade antioxidante, a par de um enriquecimento mais rápido em compostos fenólicos da madeira. Por outro lado, a atividade antioxidante, o teor de compostos fenólicos totais e as concentrações de compostos fenólicos de massa molecular baixa determinadas por HPLC apresentaram uma correlação positiva significativa. Os resultados demostraram também que a tecnologia alternativa pode ser usada como um método válido para envelhecer aguardente vinica, uma vez que permitiu obter uma bebida envelhecida de qualidade, através de um processo mais económico e ambientalmente sustentável. Observou-se ainda um efeito sinérgico com a madeira de castanheiro.

Palavras - chave

Aguardente vinica – atividade antioxidante – DPPH – tecnologia de envelhcimento – micro-oxygenaçao

Resumo alargado

Durante o processo tradicional de envelhecimento, o destilado vìnico sofre importantes alterações químicas devido à ocorrencia de múltiplos fenómenos fisico-quimicos, em que destaca a extração de compostos de madeira. Estes fenomenos são influenciados por diversos factores, tais como a espécie florestal utilizada, o nível de queima, a dimensão do barril, e as condiçoes de cave de envelhecimento.

Na tecnologia tradicional de envelhecimento recorre-se a barris de madeira. Pese embora a elevada qualidade da aguardente envelhecida assim obtida, trata-se de um processo demorado, oneroso, em que há imobilização de capital em madeira e em aguardente por um período prolongado, implicando também perda de aguardente por evaporação e utilização de uma grande quantidade de madeira que é um recurso natural limitado. Considerando que a mudança climática é uma emergência hoje em dia, é importante reduzir as emissões de gases com efeito estufa para limitar esse problema, e assim os investigadores estão a pesquisar uma tecnologia de envelhecimento nova e mais sustentável, baseada na utilização de menor quantidade de madeira. Por outro lado, utilizando depósitos de aço inoxidavel, consegue-se reduzir o espaço ocupado, bem como o tempo necessário face à quantidade de destilado que se pretende envelhecer. Para auxiliar o processo e torná-lo mais rápido, utiliza-se micro-oxigenação.

Neste trabalho pretendeu-se efetuar a comparação das duas tecnologias de envelhecimento: tradicional, em barris de madeira de 250 L; alternativa, utilizando depositos de aço inoxidavel de 1000 L com aduelas de madeira no interior sob o efeito de micro-oxigenação. O uso de diferentes tipos de madeira também foi estudado (carvalho Limousin e castanheiro), uma vez que os compostos fenólicos encontrados na aguardente envelhecida derivam principalmente da madeira em contato com o destilado.

A atividade antioxidante da aguardente vinica obtida com os diferentes tipos de envelhecimento foi avaliada pelo método DPPH, por determinação espectrofotométrica.

Os resultados obtidos evidenciaram que a tecnologia alternativa induziu uma maior atividade antioxidante, a par de um enriquecimento mais rápido em compostos fenólicos da madeira. Por outro lado, a atividade antioxidante, o teor de compostos fenólicos totais e as concentrações de compostos fenólicos de massa molecular baixa determinadas por HPLC apresentaram uma correlação positiva significativa. Os resultados demostraram também que a tecnologia alternativa pode ser usada como um método válido para envelhecer aguardente vinica, uma vez que permitiu obter uma bebida envelhecida de qualidade, através de um processo mais económico e ambientalmente sustentável. Observou-se ainda um efeito sinérgico com a madeira de castanheiro.

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List of abbreviations

AA: Antioxidant activity

Af: Final absorbance.

Ai: Initial absorbance

B: Traditional technology

C: Chestnut wood

CIELab: Colour space CIE L*a*b*

DNA: DesoxyriboNucleic Acid

DPPH: 2,2-diphenyl-1-picrylhydrazyl

$F^{\cdot}$: Antioxidant molecule

FH: Antioxidant compound

HDL: High-density lipoprotein

$HO^{\cdot}$: Hydroxyl radical

HOCl: Hypochlorous acid

HPLC: High Performance Liquid Chromatography

L: Limousin oak wood

LDL: Low-density lipoprotein

LMWC: sum of low molecular weight phenolic compounds quantified by HPLC

M: Mixture of 50% of chestnut wood and 50% of Limousin oak wood

NO: Nitric oxide

O_2^-: Superoxide anion

$ONOO^-$: Peroxynitrite

PC1: First principal component

PC2: Second principal component

PCA: Principal Component Analysis

PDO: Protected Designation of Origin

ROO: Hydroperoxyl radical

RSD: Relative standard deviation of repeatability

SD: Standard deviation

T: Alternative technology

TPC: Total phenolic content

Trolox: 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid

1. INTRODUCTION

The French paradox showed that, with the same risk factors, mortality due to cardiovascular diseases was lower in France than in other Northern European countries. This situation was explained by the Mediterranean diet followed in France, which includes foods like fresh vegetables, cheeses, wine and oil (Renaud and De Lorgeril, 1992).

The antioxidant properties of spirit wines aged in wood is ascribed to the chemical composition, particularly to the phenolic compounds. Since these features are determined by some factors ruling the ageing process, is important to analyse their impact.

During the traditional ageing process, the fresh distillate undergoes important chemical modifications through the direct extraction of the wood constituents, the reaction between the distillate and the wood, the decomposition of the wood biopolymers, the chemical reactions involving only the distillate compounds, those involving only the wood compounds and the oxidation reactions. It was proved that the chemical composition and the concentration of the phenolic compounds varies according to many factors such as the botanical species of the wood (Belchior *et al*., 2001), the toasting level (Canas *et al*., 1999; Belchior *et al*., 2001), the barrel size (Canas *et al*., 2008b) and cellar environment (Cantagrel *et al*., 1992).

The traditional ageing technology establish to age the fresh distillate in wooden barrels, but it is expensive in terms of quantity of wood, production of barrels, space, and ageing time. Considering the climatic change that is an emergency nowadays, it is important to reduce the greenhouse' emission to limits the effect of this problem, and therefore the researchers are looking for more sustainable ageing technologies (requiring less wood and thus contributing to safeguard forest ecosystems).

Therefore, alternative ageing technologies have been investigated in order to obtain a sustainable ageing process; that is, production of a high quality aged wine spirit in a short time, with lower costs and lower quantity of wood used. These alternative technologies provide to store the fresh distillate into the stainless steel tanks to reduce the space, the utilization of staves inside the tanks to allow the release of phenolic compound into the distillate, reducing the ageing time, and the use of micro-oxygenation to help the oxidation reactions.

In this context, several studies were already performed (Canas *et al*., 2016; Canas *et al*., 2019) but so far, to the best of our knowledge, the antioxidant activity has never been studied.

The present work is framed in the Project CENTRO-04-3928-FEDER-000001, which aims to contribute to broadening the knowledge on a new technology using staves and micro-oxygenation applied to the wine spirit kept in stainless steel tanks comparing it with the traditional technology,

and to increase the production of wine spirits, limiting costs and harmful effects on the environment. For this purpose, micro-oxygenation was applied to wine spirit stored in stainless steel tanks (1000 L) with staves of different types of wood (chestnut, Limousin oak, mixture of chestnut and Limousin oak). Comparison is made with the same wine spirit aged in wooden barrels (250 L) with the same kinds of wood.

2. LITERATURE REVIEW

2.1 Wine spirit

Wine spirit is produced in several countries of the world. Globally, wine spirit (with 2.0 billion litres) is the 5th largest category of spirits drink (20.0 billion litres in total) (Tsakiris *et al*., 2013).

In Portugal, there are six PDOs (Protected Designation of Origin) for Wine spirit: *Vinhos Verdes*, *Douro*, *Bairrada*, *Lourinhã*, *Tejo* and *Alentejo* – Figure 2.1. The region of *Lourinhã*, due to soil and climate conditions, is an exclusive denomination for wine spirit (Decreto Lei No 323/94), such as the *Cognac* and *Armagnac* regions in France.

Figure 2.1 - Wine spirit's Protected Designation of Origin. Source: IVV (2017).

Wine spirit is a spirit beverage obtained exclusively by the distillation of wine, fortified wine, wine possibly with the addition of wine distillate or by re-distillation of a wine distillate with the result that the product retains the aroma and taste of the above-mentioned raw material. A certain period of ageing in oak wood containers is usually done before marketing. Alcoholic strength of the end product must not be less than 37.5% volume (Regulation EC 787/2019).

The distillation is an ancient technique whose exact age is still unknown today. It is believed to date from the Chineses (3.000 a.C). It seems that the first wine was distilled in France by Arnaud de

Villeneuve, in the XV century, who called the product obtained "*eau-de-vie*" or water of life (Leauté, 1990).

The distillation aimed to increase the concentration of ethanol and the aromatic constituents already present in the wine. During the ageing, the sensory properties acquired (colour, aroma and taste) mainly derive from the compounds extracted from the wood and their reactions with the compounds of the wine distillate. Hence the wine spirit acquires aromatic and gustatory complexity and higher overall quality (Tsakiris *et al*., 2013).

For this reason, the quality of the raw material, the winemaking and the distillation processes as well as the ageing conditions, are very important.

2.1.1 Wine-base for wine spirit

Grape varieties with a high yield potential are preferred for wine spirit production (Toerien, 2008). A high fixed acidity, and therefore low sugar content, is a mandatory feature to preserve the wine until distillation without sulfur dioxide (Toerien, 2008). In order to obtain a high level of acidity it would be better to harvest the grapes before complete ripeness (Toerien, 2008).

During the distillation process there is a concentration of the volatile components; for this reason, it is better to use varieties with a neutral aroma (Bertrand, 2003). For example, the use of varieties such as Sauvignon or Chardonnay is not the best option as they have low yields and high costs; they are varieties that lead to the production of wines with a strong and persistent aroma and taste (Tsakiris *et al*., 2013).

In addition, the quality of the grapes is a very important factor. Sometimes the application of pesticides is necessary to achieve this goal. For example, the *Botrytis cinerea* (a fungus that commonly infects) has a negative effect not only on the quality of the wine but also of the wine spirit as the mould odours can be detected even after distillation and ageing (Tsakiris *et al*., 2013).

Grapes can be harvested mechanically or manually; it is important, however, that the grapes arrive in the cellar as soon as possible and without crushing to avoid fermentation under uncontrolled conditions, as for the production of wine to be consumed as such.

The best wine spirits are obtained from wine with high fixed acidity, low alcohol content, coming from grapes with good sanitary quality, free from oxidation and without sulfur dioxide (Belchior, 1987).

Red and white grape varieties are suitable to make wine for the distillation, but it should be produced by the white wine classic technology. Indeed, the stalks are removed from the grapes that are crushed and pressed thereafter. Few phenolic compounds, especially tannins, should be release

from the solid parts of the bunch. The discontinuous presses are the best option compared to the continuous one because the continuous press increases the sediments of the pomace, which increase the aromatic complexity of the wine and, secondly, of the distillate (Jurado *et al*., 2007).

After pressing, there must be the minimum possible time for the fermentation to start, as the absence of sulfur dioxide does not protect the must from oxidation and microbiological spoilage. The addition of pectolytic enzymes into the must can be used to facilitate clarification but should be avoided as they increase the methanol content in the distillate (Bajard-Sparrow *et al*., 2007).

2.1.2 Alcoholic fermentation

During fermentation, many volatile compounds are formed which are susceptible to oxidation. Since the use of sulfur dioxide is discouraged due to the fact that it can appears in the wine spirit, as mentioned above, which depreciates it or give rise to mercaptans, which have unpleasant aromas that damage the wine spirit (Belchior *et al*., 2015), it is necessary to control the temperature during fermentation (17-20°C).

2.1.3 Time between fermentation and distillation

Distillation can take place immediately after the winemaking process, or up to 5 months after (Tsakiris *et al*., 2013). The alcohol content does not decrease over time, unlike the content of esters, which are compounds that have properties of aromatic interest (such as isoamyl, acetate, hexyl acetate, phenylethyl acetate, ethyl caproate, ethyl caprylate, ethyl caprate and ethyl laurate). An undesirable increase in ethyl acetate, ethyl lactate, diethyl succinate, acetaldehyde (ethanal) and acetic acid of the wine-base is usually observed simultaneously (Cantagrel, 2003). Therefore, it would be preferable to carry out the distillation soon after winemaking.

2.1.4 Distillation

The aim of the distillation is to increase the content of ethanol and the volatile constituents present in the wine obtained from the grapes, or that form during the vinification process (Tsakiris *et al*., 2013).

Distillation is a technique that allows the separation of alcohol and volatile compounds contained in the wine (Belchior, 1987; Leauté, 1990; Cantagrel, 2008; Garreau, 2008). It consists of heating the wine to the boiling point of the volatile constituents and condensing the released vapors. During the distillation there are variations of existing volatile compounds in wine (Leauté, 1990). Since the

volatility of the compounds is related to their boiling points, the rate with which the volatile compounds of the wine are released to the hydro-alcoholic steam varies according to the different chemical group: alcohols, aldehydes, ketones, esters, nitrogen compounds, others.

There are two types of distillation: continuous or batch. Normally, continuous distillation takes place in a column still, while batch distillation takes place in an alembic. According to the Reg EC 787/2019, the distillate can reach a maximum alcohol content of 86% v/v, to retain some of the volatile compounds of the raw material.

The different distillation technologies lead to the production of distillates with different characteristics. In particular, *Cognac* results from a double distillation process in "Charentais" alembic, and *Armagnac* is obtained in a single column still. In Portugal, the distillation take place in the alembic or column still (Belchior, 1987), depending on the region and the producer.

2.1.4.1 Bach distillation

Concerning the alembic, it comprises a boiler, a hat, a swan's neck and a condenser. A pre-heater is an optional device – Figure 2.2.

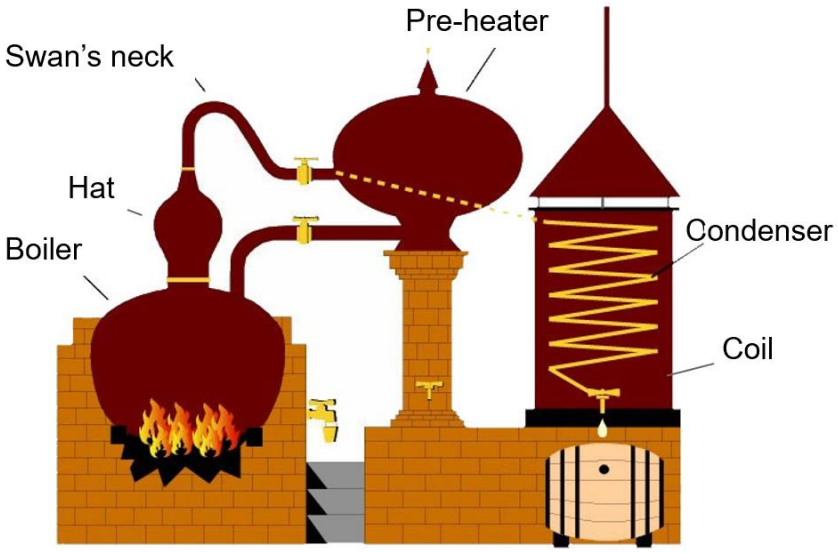

Figure 2.2 – Scheme of an alembic Charentais.

The boiler is the vessel that receives the wine and heats it evenly (Cantagrel, 2008), accumulating heat (Belchior, 1987). It is the main part of the alembic.

The hat is just above the boiler. Its shape and volume determine concentration, selection and separation of the different volatile compounds of the wine (Cantagrel, 2008). This part of the alembic collects the vapours formed in the boiler, allowing a partial condensation of some volatile compounds (with lower volatility), that return to the boiler where they are re-distilled (Leauté, 1990) improving the separation of the wine components.

After the hat, the vapours go into the swan's neck, which has the purpose of conducting them from the hat to the coil (Cantagrel, 2008).

The pre-heater is an optional structure; its main function is to recover the heat. The hot steam from the distillation allows pre-heating the wine to be used in the next distillation (Cantagrel, 2008).

The coil is formed by a cylindrical spiral tube, immersed in water, inside the condenser. The initial part of the coil has a wider diameter to facilitate condensation, and progressively decreases until it reaches the end of the condenser (Leauté, 1990).

The condenser is a tank with water that allows the condensation of the distillate. During condensation, the reaction of copper with sulfur compounds and fatty acids leads to the formation of insoluble compounds (Cantagrel *et al*., 1990) which are removed through filtration out the alembic.

The alcoholmeter allows the alcoholic strength assessment of the distillate to check the distillation progress (Leauté, 1990; Cantagrel, 2008).

The distillation in alembic requires a double distillation for the enrichment in alcohol (higher yield) and increase of purity (higher quality of wine spirits). The first distillation, which lasts 6-7 hours, depends on the alcoholic degree of the wine, gives a heart fraction of about 28 to 32% v/v. Heads are re-distilled with the succeeding batch of wine. The tails are discarded. The heart is re-distilled for 15 hours; during the second distillation the heads and the tails are commonly re-distilled with the succeeding batch of wine to take the ethanol. The distillate became 70-86% v/v (Belchior *et al*., 2015).

Batch distillation produces a more aromatic final product than the continuous one.

2.1.4.2 Continuous distillation

The continuous distillation system, on the other hand, consists of a column containing several overlapping plates in which the different volatile compounds of the wine are separated – Figure 2.3.

The wine enters by the upper part of the column, covering all the distillation plates until it reaches the base. Outside the column there is a boiler that produces a flow of steam, which is responsible for the partial evaporation of wine when it flows upwards the column through the distillation plates.

During the transition between the different plates, the wine release the volatile compounds. The liquid part descends along the column, while the gaseous part is led to a condenser from which the distillate will come out (Lafon *et al*., 1973; Belchior, 1987; Garreau, 2008). Each plate behaves as a small alembic because evaporation and condensation phenomena occur simultaneously. Once the equilibrium (temperature, flow rate and alcohol content of the distillate) is reached, there is a continuous wine inlet and a continuous distillate outlet, and the distillate is collected in containers.

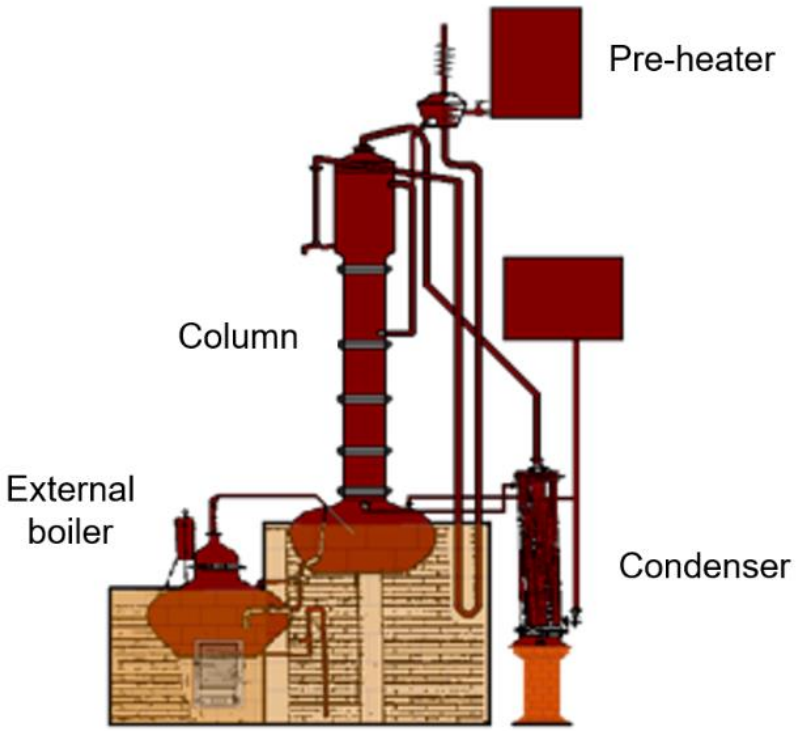

Figure 2.3 – Scheme of a column still.

Continuous distillation is considered economically advantageous (Lafon *et al*., 1973; Garreau 2008), as it allows the distillation of large quantities of wine in a shorter time, although with less aromatic and richness than the *Charentais* system (Lafon *et al*., 1973).

The alembics and columns are made from copper as it has different advantages: it is malleable, it is a good conductor of heat, it resists to corrosion from the fire and the wine, it is a catalyst for favourable reactions between wine and components, it reacts with sulfur components and fatty acids forming insoluble salts that precipitate (Leauté, 1990; Belchior *et al*. 2015).

2.1.5 Ageing

According to the European legislation (Regulation (EC) No. 787/2019), the wine spirit can be aged for at least one year in wood containers with a capacity higher than 1000 L or for at least six months in wood containers with a capacity lower than 1000 L. For wine spirits with Protected Designation of Origin, the ageing period is at least one year for *Armagnac* (Décret No. 2009-1285), and two years for *Cognac* (Décret No. 2009-1146) and *Lourinhã* (Decreto Lei No. 323/94).

Regarding the ageing technology, there are two different kinds: traditional and alternative (*vide* 2.5).

During the ageing process, the wine spirit undergoes important chemical modifications so that its sensory properties improve. These changes are due to several phenomena described below (*vide* 2.2).

2.1.6 Finishing operations

Before bottling, several wine distillates from different barrels are blended. In order to obtain the desired alcohol strength, demineralised water is added. After that it could be stabilised by cold stabilisation to remove the instable components and to avoid further turbidity (Lafon *et al*., 1973). Then, the wine spirit is filtered and bottled.

2.2 From wine distillate to aged wine spirit

After distillation, the fresh wine distillate is colourless, characterised by high ethanol content with many volatile compounds, but is devoid of phenolic compounds other than volatile phenols (Caldeira *et al*., 2016). During the ageing process, the aged wine spirit acquires green/yellowish/gold/amber colour due to the phenolic compounds released from the wood into the distillate (Canas, 2017) – Figure 2.4.

Figure 2.4 – From the wine distillate to the aged wine spirit.

The colour and the composition of the aged wine spirit depends on several factors. In the traditional technology, using wooden barrels, the most important are: i) the botanical species of the wood used (Canas *et al*., 2008a; Canas, 2017); ii) the toasting level of the wood (Caldeira *et al*., 2002; Canas, 2017); iii) the ageing time (Canas, 2017); iv) the barrel size (Canas *et al*., 2008b); v) the cellar conditions, such as temperature, relative humidity and air circulation (Adana *et al*., 2005); vi) the technological operations performed during the ageing period, such as the refilling with the same wine distillate to offset the loss by evaporation and impregnation (Canas *et al*., 2002), the water addition to decrease the alcoholic strength, and stirring to homogenize the wine spirit and to enhance the extraction of wood compounds (Patrício *et al*., 2005).

Therefore, all these factors determine the quality of the final product.

The changes during the ageing of the wine spirit derives from (Canas, 2017):

- Direct extraction of wood-constituents;
- Decomposition of wood biopolymers (lignin, hemicelluloses and cellulose) followed by the release of derived compounds into the distillate;
- Chemical reactions involving only the wood extractable compounds;
- Chemical reactions involving only the distillate compounds;
- Chemical reactions between the wood extractable compounds and the distillate compounds;
- Evaporation of volatile compounds and concentration of volatile and non-volatile compounds;

- Formation of a hydrogen-bonded network between ethanol and water.

The release by the wood of the extractable compounds plays a fundamental role (Puech *et al*., 1985; Canas *et al*., 2000a; Caldeira *et al*., 2008). Moreover, oxidation reactions (Belchior *et al*., 1982; Avakiants *et al*., 1992; Monsedale and Puech, 1998; Canas *et al*., 2009; Cernîsev, 2017), which are triggered by the slow diffusion of oxygen from the space between the staves and through the wood (Moutounet *et al*., 1998; Del Álamo-Sanza and Nevares, 2014; Del Álamo-Sanza *et al*., 2016), are of great importance.

The remarkable change in the amount of phenolic compounds before and after ageing should be stressed.

The importance of phenolic compounds, which are mainly extracted from the wood, lies on their influence on the sensory properties (colour, aroma and taste) and on their many biological effects, due to their great antioxidant activity. Many studies reported the antioxidant activity of phenolic acids (Canas *et al.*, 2008a; Alañón *et al*., 2011a; Alañón *et al*., 2011b, Heleno *et al*., 2015), phenolic aldehydes, coumarins, tannins, lignans and some volatile phenols (Canas, 2017).

Chemically, these compounds have at least one aromatic ring in which at least one hydrogen is substituted by a hydroxyl group (Heleno *et al*., 2015).

Among the wood phenolic compounds, the phenolic acids, such as gallic acid, ellagic acid, syringic acid and vanillic acid are the most abundant in aged wine spirits (70%), followed by phenolic aldehydes (15%) such as sinapaldehyde, syringaldehyde and vanillin, followed by lignans (12%), phenyl ketones (3%) and coumarins (0.1%) (Canas, 2017).

2.2.1 Phenolic acids and phenolic aldehydes

The phenolic acids can be classified into two major groups, hydroxybenzoic acids (in C7) and hydroxycinnamic acids (in C9) (Shahidi and Yeo, 2016). Vanillic acid, syringic acid, ferulic acid, gallic and ellagic acid have been found in the aged wine spirits (Canas, 2017) – Figure 2.5.

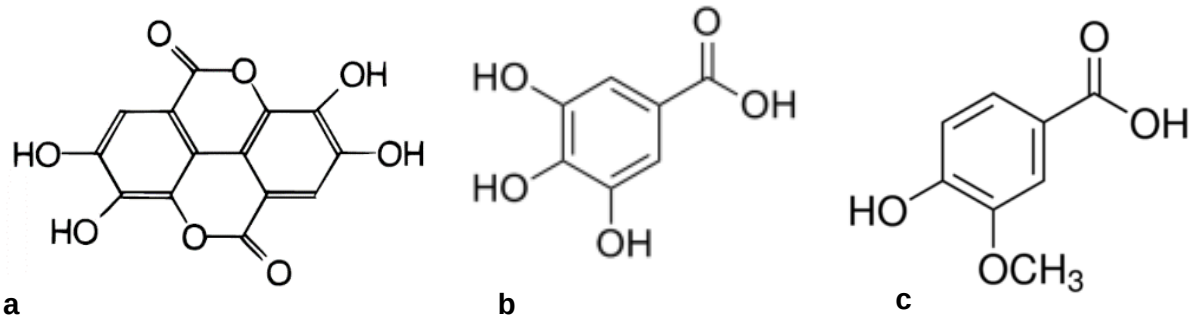

Figure 2.5 – Principal phenolic acids found in aged wine spirits; a: ellagic acid, b: gallic acid, c: vanillic acid.

Phenolic aldehydes contain both the phenolic and the aldehydic group. These compounds can be classified according to the number of carbon atoms into benzoic (C7) and cinnamic (C9); and according to the number of methoxyl groups into guaiacyl-type (mono methoxylated) and syringil-type (bimethoxylated) – Table II.1; Figure 2.5.

Table II.1 – Classification oh the phenolic aldehydes.

Component	**N° carbon atoms**		**N° methoxyl groups**	
	C7	C9	1 (mono-)	2 (bi-)
Vanillin	x		X	
Syringaldehyde	x			x
Coniferaldehyde		x	X	
Synapaldehyde		x		x

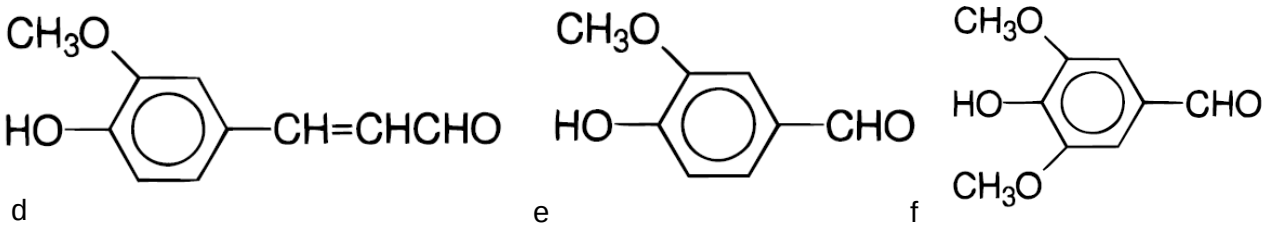

Figure 2.6 Principal phenolic aldehydes found in aged wine spirits; d: coniferaldehyde, e: vanillin, f: syringaldehyde.

The phenolic acids and phenolic aldehydes derive from lignin. They are present in oak and chestnut wood in the free and esterified forms (Canas *et al*., 2000c), being particularly important due to their implications in the physicochemical (Puech, 1984; Puech *et al*., 1985; Canas *et al*., 1999; Canas, 2003) and organoletic characteristics (Canas *et al*., 1998; Caldeira *et al*., 2008) of the aged spirits.

The concentration of the above-mentioned compounds changes according to the thermal treatment of the wood (Canas *et al*., 2000b; Canas *et al*., 2007; Alañón *et al*., 2010; Sanz *et al*., 2010; Le Floch *et al*., 2015), which also affects the permeability of wood (Hale *et al*., 1999; Acuña *et al*., 2014). When the toasting temperature is between 120–165°C, phenolic aldehydes (primarily cinnamic aldehydes such as coniferaldehyde and sinapaldehyde) increase due to the thermal degradation of lignin. When the toasting temperature is above 165°C, a more intense thermolysis process occurs, resulting in the degradation of cinnamic aldehydes into benzoic aldehydes (vanillin and syringaldehyde) (Li and Duan, 2018).

During ageing, hydroalcoholysis of lignin also contributes to the enrichment of the wine spirit in phenolic aldehydes and phenolic acids (Nishimura *et al*., 1983; Puech, 1984; Cernîsev, 2017).

2.2.1.1 The influence of different kinds of wood

Canas *et al*. (2008a) showed that the wine spirit's phenolic composition (total phenolic content, phenolic acids and hydrolysable tannins) change according to the type of wood used, the toasting level of the barrels, and the ageing time. Regardless of the toasting of wood, the wine spirit aged in chestnut wood showed greater concentration of phenolic compounds than the wine spirit aged in Limousin oak, confirming the results of previous studies (Belchior *et al*., 2001; Canas, 2003). In addition, the wine spirit aged in chestnut wood was richer in phenolic acids than the wine spirit aged in Limousin oak, except for ellagic acid. The former stood out by its greater concentration of gallic acid (Canas, 2017).

2.2.1.2 The influence of the ageing time

Ageing time has also been examined (Canas *et al*., 2008a), showing that the total phenolic content, tend to increase during the ageing, especially in the first year. In detail, this study demonstrated that ellagic acid increased during the ageing period likely by wood ellagitannins hydrolysis and direct extraction, gallic acid also increased during the ageing period, ferulic acid increased continuously with the ageing time, while vanillic and syringic acids increased up to third years and then decreased. Gallic acid content seemed to determine the evolution of the antioxidant activity of the aged wine spirits, since these two variables showed highly significant correlation (Canas *et al*., 2008a).

Moreover, the phenolic composition of beverages under ageing in wooden barrels is dynamic due to the continuous and slow oxidation (Ávila-Reyes *et al*., 2010).

2.2.2 Furanic aldehydes

Furanic aldehydes (furfural, 5-methylfurfural and 5-hydroxymethylfurfural) are formed by the thermal degradation of cellulose and hemicelluloses, and their concentrations are positively correlated with the toasting level (Caldeira *et al*., 2002; Canas, 2017). Besides, furfural is already present in the wine distillate (Caldeira *et al*., 2010). Regardless of the ageing conditions, such compounds have been also found in the aged wine spirits (Canas *et al*., 2003).

2.3 Antioxidant activity

The French paradox showed that, with the same risk factors, mortality due to cardiovascular diseases in France was lower than in other Northern European countries. This situation was ascribed to the Mediterranean diet followed in France, which includes foods like fresh vegetables, cheese, wine and olive oil (Renaud and De Lorgeril, 1992). All these foods share strong antioxidant properties (Vinson *et al*., 2001; Fernandez-Pachon *et al*., 2004; Cicerale *et al*., 2011; Rashidinejad *et al*., 2013).

In recent years, the interest about antioxidant power in food is growing. There are many studies on the antioxidant activity of food; often, the red wine aged in wood is considered as a source of compounds with antioxidant properties (Ivanova-Petropulos *et al.*, 2015). Nowadays, some studies show the antioxidant properties of wine spirit aged by the traditional technology using wooden barrels (Canas *et al*., 2008a; Schwarz *et al*., 2009). In fact, *Armagnac*, *Cognac* and other aged wine spirits are rich in phenolic compounds due to their ageing in wooden barrels (Da Porto *et al*., 2000; Umar *et al*., 2003ab; Canas *et al*., 2008a; Schwarz *et al*., 2009).

The antioxidant properties of the wine spirits aged in wood are attributed to the concentration of phenolic compounds (Da Porto *et al*. 2000; Umar *et al*., 2003a,b; Canas *et al*., 2008a), which depends on the above-mentioned factors.

2.3.1 Antioxidant activity of foods and plants

Recently, people are increasingly thinking about limiting the use of medicines to promote the consumption of healthy food or plants, preventing some diseases or curing them. For example, in order to minimise the antibiotic resistance, antimicrobial agents deriving from plants are often used (Stanković *et al*., 2016).

The work of Stanković *et al*. (2016) about the antioxidant action of eight plants revealed that the most abundant antioxidant compounds in the plant material are of phenolic nature.

The study conducted by Zhang *et al*. (2013) showed how important it is the investigation of the antioxidant activity of the phenolic components of the tea for quality control and how the antioxidant activity of different teas (green, black, oolong and white tea) varies according to the production process.

Dragović-Uzelac *et al*. (2007) highlighted Cornelian Cherry and Sour cherry cv. Marasca as the cherry varieties with the greatest antioxidant activity.

Tuberoso *et al*. (2013) classified the Cannonau wine (widespread in Sardinia) and myrtle liqueur as very promising nutraceutical foods. They also suggest that isolation and purification of phenolic

compounds of such beverages can be of great interest towards the production of supplementary antioxidants.

Serreli *et al*. (2017) studied the antioxidant activity of *Nuragus* wines (a part of Italian PDO white wines) and the data can help the wine producers to promote this monovarietal wine as a valid complement associated with the Mediterranean diet.

2.3.2 Oxidative stress

The diet including foods with antioxidant properties is essential to assure the cellular redox balance due to the continuous formation of oxidants during metabolic processes in the human body. Indeed, during aerobic metabolism, different species of reactive oxygen can be formed, some of which are radicals deriving from reduction and oxidation reactions (Borguini, 2006). When there is an imbalance between their production and elimination in the human body, oxidative stress occurs, and biological molecules such as proteins, lipids, DNA are negatively affected, leading to cellular lesions (Halliwell, 1999; Valko *et al*., 2007). Oxidative stress contributes to cellular ageing, accelerating the development of various diseases, such as cardiovascular diseases, cancer, neurodegenerative diseases, diabetes and the decline of the immune system (Roussel, 2002). In addition, there are many factors favouring the oxidative stress, such as drugs, pollution, malnutrition, eating disorders, alcohol, tobacco and radiation.

Due to its content in phenolic compounds, mainly flavonoids and phenolic acids, wine can be considered as a source of elements capable of preventing diseases associated with oxidative stress (Cataneo *et al*., 2008; Golluke *et al*., 2009; Maier *et al*., 2009).

2.3.3 Free radicals

Free radicals are defined as molecules or molecular fragments containing one or more unpaired electrons in atomic or molecular orbitals (Valko *et al*., 2007).

The oxygen reactive species (superoxide anion O_2^{-}, hydroxyl radical $HO^{\cdot}$, hydroperoxyl radical ROO, nitric oxide NO and lipidic radicals) and non-radicals (singlet oxygen, hydrogen peroxide H_2O_2, peroxynitrite $ONOO^{-}$, hypochlorous acid HOCl) possess oxidant activity (Urso and Caimi, 2011).

Free radicals are chemically unstable and very reactive species due to their electronic configuration. In human body, they derive from different metabolic pathways, such as the mitochondrial respiration.

2.3.4 Correlation between antioxidant activity and phenolic compounds

The antioxidant activity of the phenolic compounds is ascribed to their redox properties. Phenolic compounds are hydrogen or electron donors, so in the presence of reducing compounds they can donate a hydrogen, breaking the chain of free radicals and become more stable O-centered radicals (Stanković *et al*., 2016; Hoyos-Arbeláez *et al*., 2017) – Figure 2.7.

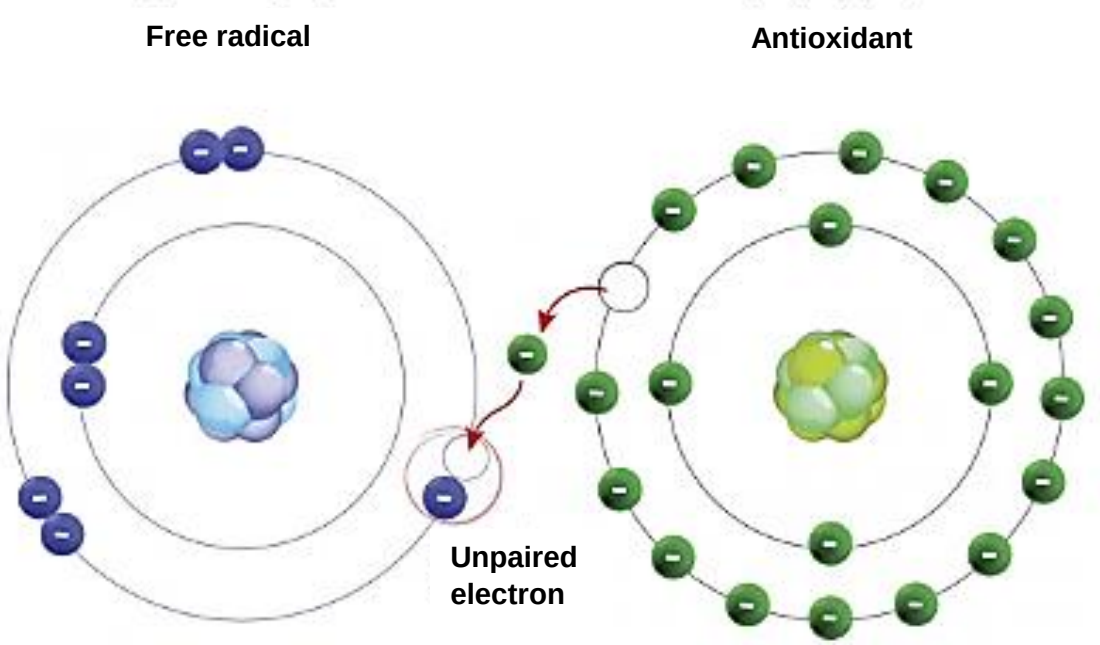

Figure 2.7 – Reaction between free radical and antioxidant molecule. Source: Villines (2017).

Therefore, the antioxidant activity of phenolic compounds is partially due to their ability to scavenge free radicals. The structure of phenolic compounds is the key determinant of radical scavenging. In the case of phenolic acids, the antioxidant activity depends on the number and position of the hydroxyl group in relation to the carboxylic functional group; their antioxidant activity increases with the increase in the degree of hydroxylation. For example, the trihydroxyl gallic acid shows higher antioxidant activity than gallic acid (Balasundram *et al*., 2006).

Some works evidence the pharmacological properties of phenolic aldehydes and phenolic acids as antioxidants, antiviral, antibacterial, anti-inflammatory, anti-hepatotoxic, anti-thrombotic and anti-cancer (Scalbert and Williamson, 2000; Okuda, 2005; Al Awwadi *et al*., 2007; Terra *et al*., 2007). Antioxidant activity is mainly due to its redox properties, such as inhibition of lipid peroxidation and oxidative DNA damage, playing an important role in combining and neutralizing free radicals (Degáspari and Nina, 2004; Saura-Calixto and Goñi, 2006; Vekiari *et al*., 2008).

As Canas (2017) showed that some phenolic acids found in aged wine spirits had a significant correlation with the antioxidant activity; from the highest to the lowest: ellagic acid, gallic acid, and

vanillic acid. These results are in agreement with those of Ziyatdinova *et al*. (2014), who demonstrated the strong antiradical activity of ellagic and gallic acids even at low concentrations.

Zafrilla *et al*. (2001) reported that ellagic acid promotes greater scavenging of free radicals hydroxide and peroxide, being therefore very effective against oxidative stress when compared with antioxidants, such as vitamin C and E (Hassoun *et al*., 1997).

2.3.5 Antioxidant activity correlated to the ageing conditions

Canas *et al.* (2008a) found that the wine spirit's antioxidant activity changed according to the type of wood used in its ageing. Specifically, they observed that the antioxidant activity in the wine spirit aged in oak barrels was two times less than in the wine spirit aged in chestnut barrels. This difference was assigned to the richness of the later in gallic, ellagic and vanillic acids (Canas *et al.*, 2008a).

Concerning the ageing time, several authors (Goldberg *et al*., 1999; Da Porto *et al*., 2000; Alonso *et al*., 2004; Canas *et al.*, 2008a) reported that the antioxidant activity and the total phenolic content tend to increase over the time.

A non-significant effect of the toasting level of the wood (light, medium and heavy) on the wine spirit's antioxidant activity was pointed out by Canas *et al.* (2008a), despite the significant influence of this ageing condition on the phenolic composition of the wine spirit (Canas, 2017).

2.4 Wine spirit consumption and human health

This topic is of great interest in spirit drinks as the harmful effect of the high alcohol strength on the consumer's health can be offset by the intake of bioactive compounds. These benefits mainly derive from the wood contact so they are expected in beverages such as wine spirit and whiskey, and not in un-aged alcoholic beverages such as gin or vodka (Goldberg et al., 1999).

Many studies established the relationship between alcoholic beverages and health. Despite numerous doubts, there is scientific evidence on the beneficial effects of moderate wine consumption on cardiovascular diseases, diabetes and osteoporosis; such positive influence is also likely in neurological diseases and longevity. However, the findings are not consistent on the effects on cancer (Artero *et al*., 2015).

The beneficial effect of moderate alcohol consumption on various cardiovascular pathologies is associated with an increase in HDL (High-Density Lipoprotein) which favours the absorption of antioxidant phenolic compounds in the intestine.

2.4.1 Antioxidant activity of the wine spirit and its beneficial effects on health

The Dietary Guide Lines for Americans 2010 recommends moderate alcohol consumption, up to one drink per day for women and two per day for men (Sus, 2011).

Alcohol, as several studies show, has the ability to increase the concentration of high-density lipoprotein (HDL), which inhibit platelet aggregation and limit blood coagulation, and also reduce low-density lipoprotein (LDL) responsible for cholesterol (Goldberg *et al*., 1999; Schroder *et al*., 2006). Because of these effects, some studies reported that the wine spirit's antioxidant activity can have beneficial effects on health. The *in vivo* study of Duriez *et al*. (2001) showed that after drinking *Cognac*, the concentration of phenolic acids, in particular ellagic acid, increased the blood plasma content and antioxidant capacity. The study made by Umar *et al*. (2003b) showed how *Armagnac*, administered to rats, caused a decrease in platelet aggregation on the arteriovenous thrombus, reducing the onset of thrombosis. But, in order to assess whether the beneficial effect on platelet aggregation and thrombosis is determined by alcohol in general or by certain alcoholic drinks, Umar *et al*. (2005) compared the effects of small amounts of *Armagnac* and vodka in humans, noticing a reduction in platelet aggregation during and after consumption of *Armagnac* rather than vodka. These studies, thus, highlight the synergism between phenolic compounds, particularly with ellagic acid.

2.4.2 Negative effects associated with wine spirit consumption

In spite of the benefit aforementioned, the spirit drinks have a negative effect in human health due to its high alcohol content.

Indeed, the studies on the adverse effects of alcohol consumption should not be overlooked, which highlight the strong relationship between the amount of alcohol consumed and the increase in mortality, mainly due to liver disease and cardiovascular disease (Ferreira and Weems, 2008).

Schroder *et al*. (2006) conducted an epidemiological study, in which they found that increased alcohol consumption was associated with high levels of LDL oxidation in the plasma of the population studied. Addolorato *et al*. (2008) studied the influence of alcohol consumption on healthy humans, observing the decreased energy levels (ATP) of the decreased blood plasma and, consequently, the antioxidant state. In this sense it is important to highlight that the effects of phenolic compounds in the body are more effective if associated with regular consumption, since their action develops over time, providing greater protection than the consumption of large quantities of irregular shapes (Addolorato *et al*., 2008).

Besides, a compound with antioxidant properties, such as gallic acid, depending on their concentration and the presence of metal ions, such as copper or iron, can act as a pro-oxidant in the body. However, this pro-oxidant effect of gallic acid has also been reported as having an anti-proliferative action on cancer cells (Babich *et al*., 2011).

2.5 Wine spirit's ageing technologies

The previous research studies were made on the traditional technology using wooden barrels, proving the nutraceutical quality of the aged wine spirit's obtained.

Lately, the research has been focused on the alternative technologies to obtain high quality wine spirit, but reducing costs (Schwarz *et al.*, 2014), thus limiting the use of wood, for both economic and environmental advantages, and reducing time of processing. Most of the studies were based on the ageing of wine spirits in stainless steel tanks with staves.

Canas *et al*. (2016) compared the use of traditional technology (barrels) with the new technology (stainless steel tanks with staves). The quantity of staves was calculated in order to reproduce the surface area to volume ratio of barrels. The dry extract, calculated according to the OIV method (2009), showed that the aged wine spirit in stainless steel tanks with staves had a lower dry extract than the wine spirit aged in barrels. The total polyphenol index was calculated through spectrophotometric analysis (Cetò *et al.*, 2012) showing lower values in wine spirit aged in stainless steel tanks with staves. Low molecular weight compounds were determined using High Performance Liquid Chromatography (HPLC) (Canas *et al.*, 2003), showing that 5-methylfurfural was the only compound present in greater quantity in wine spirit obtained by the alternative technology, rather than the traditional method. However, at the end of the ageing process the colour determined by the CIELab method, was more intense and evolved in the wine spirits aged in stainless steel tanks with staves than in those aged in barrels.

In order to explain this behaviour, the authors studied the influence of the toasting level of the wood. They did not find a key role of this factor to explain the aforementioned differences. In addition, their results were not in agreement with those of Jourdes *et al*. (2011). These latter authors reported that the extraction from the pieces of wood was favoured due to a faster absorption of the liquid through them, followed by an easier penetration through the xylem vessels, facilitating the release of these compounds.

2.5.1 Micro-oxygenation

The alternative ageing aims to reproduce the traditional one, while assuring a faster and less costly process. The most widespread technology consists of adding wood pieces (staves, cubes, tablets, chips, among others) to the beverage stored in stainless steel tanks.

Oxygen plays an important role during winemaking and ageing, as Rivero-Pérez *et al*. (2008) report about wines. The oxygen influences the phenolic composition, and indirectly influences the sensory properties (namely colour, aroma and astringency). To simulate the oxygen transfer occurring in the barrel, the micro-oxygenation technique developed by Ducournau and Laplace (Parish *et al*., 2000)

has been applied to optimize this novel technology. Several studies made on red wine (Gómez-Plaza and Cano-López, 2011), wine vinegar (Guerrero *et al*., 2011), and cider brandy (Rodríguez *et al*., 2013) report it. Concerning the wine spirit, few works report the phenolic and colour responses to the ageing with wood pieces (Canas *et al*., 2016; Cruz *et al*., 2012; Schwarz *et al*., 2011), only one was published on the effect of micro-oxygenation applied during the ageing of this beverage (Canas *et al*., 2019).

For this reason, Canas *et al.* (2016) stated that the chemical differentiation of the aged wine spirit can be determined by other factors such as its oxidation state. According to Avakiants (1992), oxygen is a fundamental element in reactions occurring during ageing, involving phenolic and furanic compounds.

The above-mentioned studies are the basis of the present work, framed in the Project CENTRO-04-3928-FEDER-000001, which intends to investigate the effects of micro-oxygenation during the ageing process, in order to achieve high quality wine spirit by a more sustainable ageing. For this purpose, staves and micro-oxygenation were applied to wine distillate kept in stainless steel tanks and their effect was compared to that of barrels during the first 12 months of ageing.

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Wine spirits

The same wine distillate produced by the Adega Cooperativa da Lourinhã in 2017 (alcohol strength, 77.4% v/v; pH, 5.44; total acidity, as acetic acid, 0.13 g/hL of absolute ethanol) was aged according to the traditional ageing technology (B), in 250 L wooden barrels, with different types of wood and according to the alternative technology (T), using micro-oxygenation in 1000 L stainless steel tanks with staves of the same kinds of wood; three replicates were used for wooden barrels and two replicates were used for stainless steel tanks – Figure 3.1.

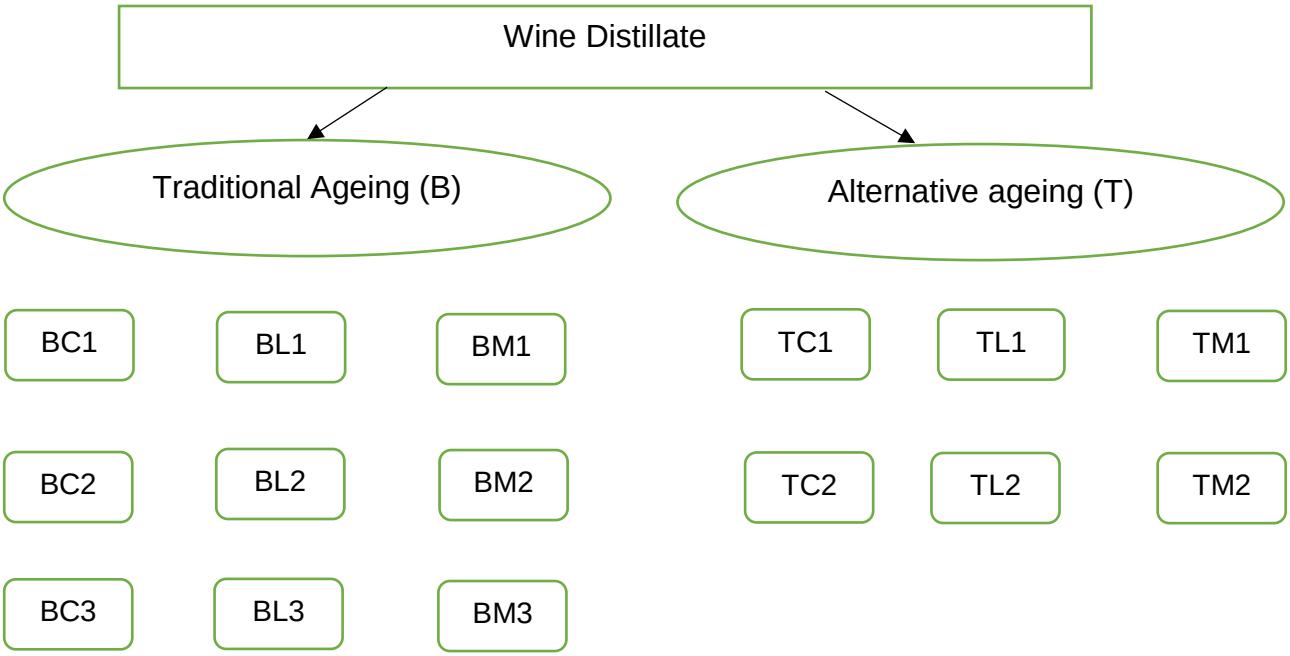

Figure 3.1 – Experimental design scheme.

The kinds of wood used were: chestnut, *Castanea sativa* Mill. (C), Limousin oak, *Quercus robur* L. (L), and a mixture of 50% of chestnut and 50% of Limousin oak (M).

The wood staves and the barrels, with medium plus toasting level, were produced by the J. M. Gonçalves cooperage (Palaçoulo, Portugal). The wood was subjected to an average temperature of 240 °C for 90 minutes. The toasting thickness varied for barrels and staves, 0.6 cm and 1.8, respectively. The staves were heated in an industrial oven, while the barrels were heated over a fire of wood offcuts, under strict control of the wood's temperature to guarantee the same degree of toasting level.

Concerning the alternative technology, the quantity of the wooden staves (91 cm length x 5 cm width x 1.8 cm thickness) insert into the stainless steel tanks was calculated to reproduce the surface to volume ratio of a 250 L wooden barrel (85 cm^2 / L), to be comparable.

The tanks were provided with micro-oxygenation during the ageing period, simulating the amount of oxygen entering through the barrels. Using a multiple diffuser micro-oxygenator (VISIO 6, Vivelys, France) with ceramic diffusers, pure oxygen (X50S Food, Gasin, Portugal) was supplied at a flow rate of 2 mL/L/month. The flow rate was selected based on the available data for the oxygen transfer rate in Limousin oak new barrels – 1.75-2.8 mL/L/month (Canas *et al*., 2019).

The experiment was established on 29th November 2017 at Adega Cooperativa da Lourinhã (Figure 3.2). The tanks and the barrels were under similar cellar conditions.

Figure 3.2 – Adega Cooperativa da Lourinhã, Portugal.

3.1.2 Sampling

The 15 aged wine spirits (nine from barrels and six from stainless steel tanks) were sampled at middle height of the barrel or tank during the first year of ageing; after 8 days, 15 days, 30 days, 180 days and 365 days. Therefore, 75 wine spirits samples were collected and analysed.

3.1.3 Chemicals and reagents

2,2-diphenyl-1-picrylhydrazyl (DPPH) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Aldrich (Steinhein, Germany).

Methanol analytical grade was purchased from Merck (Darmstadt, Germany).

Ultrapure water produced by Arium Comfort equipment (Sartorius, Göttingen, Germany) was used.

3.2 Methods

3.2.1 Analytical methods

3.2.1.1 Antioxidant activity of the aged wine spirits

Among several analytical methods available, it was decided to use the DPPH method to assess the antioxidant activity of these wine spirits. This method was introduced by Blois in 1958 and has been used for wines (Brand-Williams *et al*., 1995; Mensor *et al*., 2001) and wine spirits (Da Porto *et al*., 2000; Canas *et al*., 2008a). Indeed, DPPH is a free radical known to be stable due to the delocalisation of the spare electron in the molecule, so that the molecule does not dimerise (Brand-Williams *et al*., 1995; Molyneux, 2003). In addition, Aruna (2001) considers this method to be efficient because DPPH reacts with the sample in its entirety. It can be applied to solid and liquid matrices, regardless of the sample complexity (Aruna, 2001).

DPPH has a maximum absorbance at 517 nm. It is sensitive to light, oxygen, pH and the type of solvent used (Ozcelik *et al*., 2003). When the radical is in the presence of electron donors or of hydrogen atoms, such as phenolic compounds, electronic coupling takes place so the DPPH becomes DPPH-H (Figure 3.3).

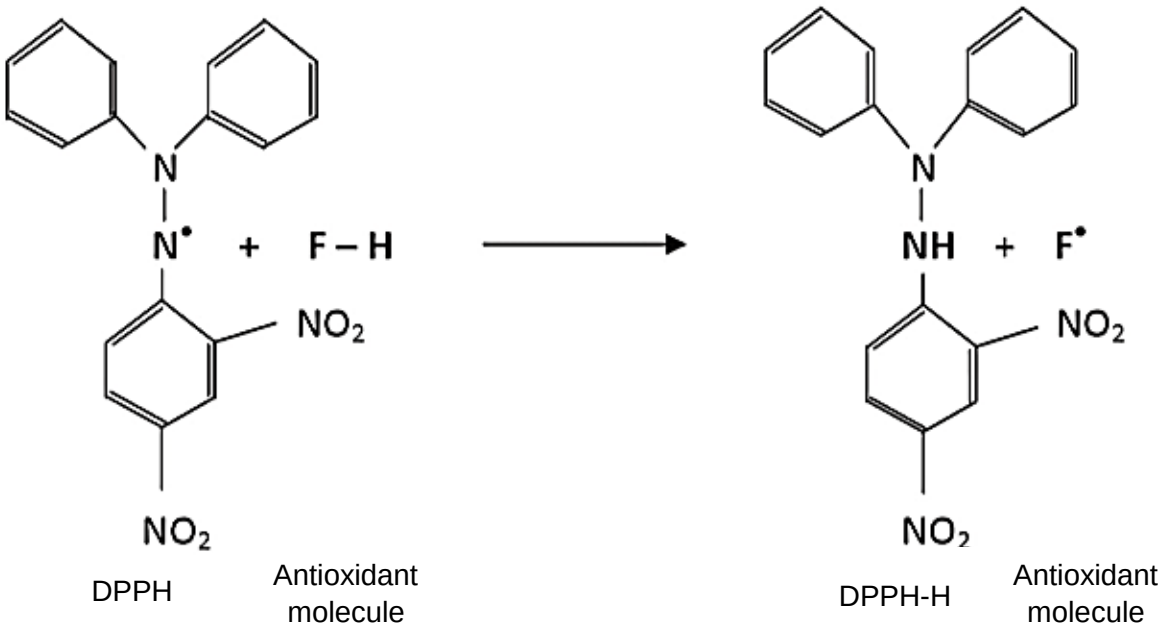

Figure 3.3 – Reaction between DPPH and an antioxidant molecule. F – H: antioxidant molecule; F·: antioxidant molecule

During the reaction the colourless DPPH-H hydrazine is formed, with a decrease in absorbance (Cano *et al*., 2002). Hence, the degree of discoloration of the DPPH solution (from purple to yellow) after the addition of the antioxidant compound (FH), is ascribed to the reducing power of the compound and, therefore, is a way to evaluate spectrophotometrically its antioxidant activity – Figure 3.4.

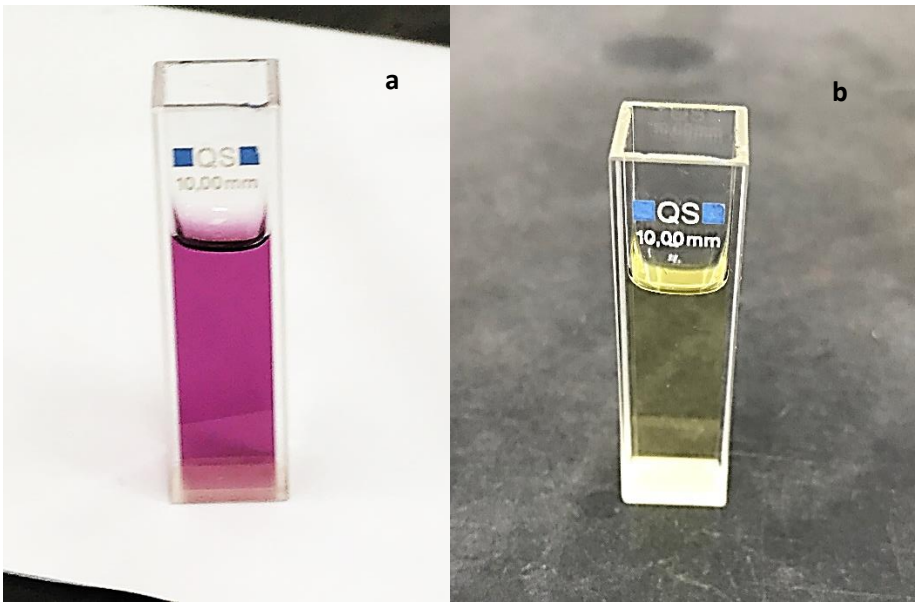

Figure 3.4 – Colour of DPPH solution with wine spirit before the reaction (a) and after the reaction (b).

The kinetics of the underlying reaction is shown schematically in Figure 3.5.

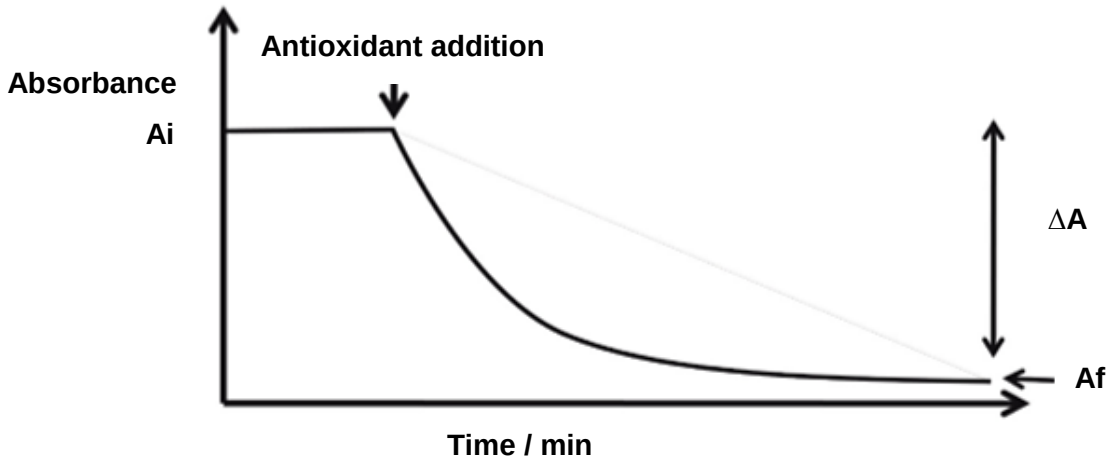

Figure 3.5 – Representative scheme of DPPH reaction with an antioxidant compound over the time.

Through the △A value, obtained spectrophotometrically after stabilizing the reaction, and applying the Lambert - Beer Law, it is possible to calculate the percentage of DPPH inhibition for the added antioxidant (Scherer and Godoy, 2009).

Since the DPPH method previously applied to wine spirits (Canas *et al.*, 2008a) is time-consuming and laborious, a new DPPH method, adapted from the one described by Ziyatdinova *et al*. (2014), was tested and then used in the wine spirits' analysis.

Briefly, the optimised method was performed as follows:

- In a quartz cell with a lid, 3 mL of methanolic solution of DPPH 8.5 x 10^{-5} M and 10 μL of methanol (control) were inserted to determine the **Ainicial**.
- Then, in the test tubes, 3 mL of methanolic solution at 8.5 x 10^{-5} M DPPH and 10 μl of wine spirit were added. The reaction between the methanolic solution of DPPH and the wine spirit takes place in the tubes, wrapped with aluminium foil to avoid the entry of light. The DPPH solution and the tubes were kept at a constant temperature of 30 ° C in a thermostatic bath, to assure the repeatability of the method. The tubes were shaken every 10 minutes for 60 minutes using a vortex.
- After 60 minutes, the DPPH solution with wine spirit was transferred to the quartz cell for spectrophotometric measurement. The absorbance reading after 60 minutes corresponds to the time required to establish equilibrium reaction, that is, the balance between the oxidized (antioxidant) and reduced (DPPH) species in the solution, which results in **Afinal**.

All the determinations were performed on a Varian Cary 100 Bio spectrophotometer (Santa Clara, USA) using 1 cm optical path quartz cells. Absorbance was measured at 515 nm.

The analysis was made in triplicate.

The percentage of DPPH inhibition, which corresponds to the antioxidant activity, was calculated according to the Equation 1.

$$\%\text{DPPH inibition} = (A_{initial} - A_{final}) / A_{inicial} \times 100 \qquad \text{Eq. 1}$$

The results were expressed in percentage of DPPH inhibition and in mM of Trolox, through a Trolox calibration curve, made under the same conditions.

3.2.1.2. Determination of total phenolic content of the aged wine spirits

Since the time available to perform this work was limited and there was high number of samples to assess the antioxidant activity, it was not possible to analyse the phenolic composition of the wine spirits under study. The project team performed the determination of total phenolic content and the determination of low molecular weight phenolic compounds of the aged wine spirits according to the methods described below. The corresponding outputs were used to study the correlations between them and the antioxidant activity (AA), as well as in the global analysis of the data.

The total content of phenolic compounds (TPC) was determined based on the absorbance measurement at 280 nm (Ribéreau-Gayon, 1970). Indeed, the phenolic compounds, having benzene nuclei, present maxima absorption of ultraviolet radiation between 280-282 nm. The total phenolic index was calculated by multiplying the measured absorbance by the dilution factor.

Determination was made in a Varian Cary 100 Bio spectrophotometer (Santa Clara, California, USA) using 1 cm optical path quartz cells.

3.2.1.3. Determination of low molecular weight phenolic compounds of the aged wine spirits

The identification and quantification of phenolic compounds was performed by HPLC. The chromatographic method used in the analysis of the wine spirit for the separation and quantification of phenolic acids (gallic, vanillic, syringic and ellagic), phenolic aldehydes (vanillin, syringaldehyde, coniferaldehyde and sinapaldehyde) was developed and validated by Canas *et al*. (2003). This is an efficient method that allows a good separation and quantification of phenolic compounds of aged wine spirits without requiring prior sample preparation.

A HPLC Lachrom Merck Hitachi (Darmstadt, Germany) was used, consisting of: quaternary pump L-7000, a column oven L-7350, a UV-vis detector L-7400, and an autosampler L-7250 and software HSM D-7000 (*Merck*) for the acquisition and treatment of data.

Calibrations curves were established with commercial standards and the results were expressed as mg/L of each compound.

3.2.2 Statistical methods

3.2.2.1 Calculation of mean, standard deviation and coefficient of variation

Since three replicates were used to analyse the antioxidant activity of each sample, the mean, standard deviation and coefficient of variation were determined for each sample analysed. The Microsoft Excel program (Microsoft, Santa Rosa, USA) was used to calculate these statistical parameters.

3.2.2.2 Analysis of Variance (ANOVA)

For the study of each factor (ageing technology, kind of wood and ageing time) a three-way analysis of variance (factorial analysis) was performed. When a significant ($P > 95\%$; $p < 0.05$), very significant ($P > 99\%$; $p < 0.01$) or highly significant ($P > 99.9\%$; $p < 0.001$) effect was observed, the means were compared through the Fisher test.

The analysis was carried out through *Statistics vs '98 edition program* (Statoft Inc., Tulsa, EUA).

3.2.2.3 Correlation analysis

In order to study the relationship between the percentage of DPPH inhibition (AA), and the total phenolic content (TPC), and the low molecular weight phenolic compounds concentrations of the wine spirits, a correlation analysis was made through Excel (Microsoft, Santa Rosa, California, USA).

3.2.2.4 Multivariate analysis

Multivariate analysis was performed to make a global analysis of the data, using NTSYSpc vs 2.10q program system developed by Rohlf in 1993 (Exeter software, New York, USA). In order to be able to graphically represent the objects (OTUs) - wine spirits - in the space defined by the analysed variables (total phenolic content, low molecular weight phenolic compounds concentration and DPPH inhibition) a Principal Component Analysis (PCA) was chosen. This analysis allows to obtain a projection in which the OTUs are designed in a system of orthogonal axes (3), resulting from the linear combination of the n axis corresponding to the n variables used (Curvelo-Garcia *et al*., 1987).

4. RESULTS AND DISCUSSION

4.1 Study of the DPPH method

In order to study the DPPH method to obtain reliable results for the antioxidant activity in a short analysis time, performance criteria were examined for parameters including practicability, linearity and repeatability (C.E.E., 1989; ISO 8466/1; Monteiro and Bertrand, 1990).

4.1.1 Practicability

Practicability is not an intrinsic characteristic of the analytical method, depending only on its purpose. Therefore, assuming that adequate standards, reagents, material and equipment are available, the simplicity and speed of the method guarantee its practicability (C.E.E., 1989). Therefore, the method is practicable because it is simple to performs and the analysis duration is short within the reaction time limits.

4.1.2 Linearity

The study of linearity was performed using methanolic DPPH solution and Trolox. Six different concentrations of Trolox solution were used, in duplicate. Regression analysis and the subsequent decomposition of ANOVA were performed; calculations were carried out by the least-squares method (analysis of variance with statistical F-test, including evaluation of the model lack-of-fit) and the regression analysis.

Results of the regression analysis are shown in Figure 4.1 and Table IV.1, and the outputs of ANOVA decomposition are presented in Table IV.2.

Table IV.1 – Analytical characteristics of the regression curves.

	Coefficients	**p value**	**95% inferior**	**95% superior**
SDa [a)]	1,593910217	0,0085	0,5072	2,6807
SDb [b)]	6,188001708	8,5429E-16	6,0316	6,3442

a - intercept; a) – standard deviation of the intercept; b – slope; b) – standard deviation of the slope.

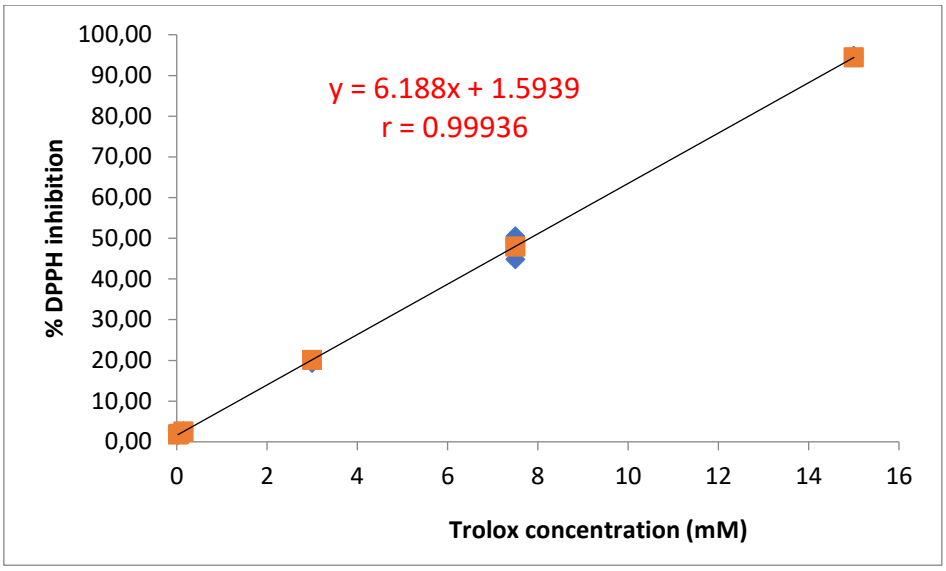

Figure 4.1 – Regression curve between the percentage of DPPH inhibition and the Trolox's concentration.

The results showed that the linear regression (Table IV.1) is the best method to establish a relationship between the percentage of DPPH inhibition and the Trolox's concentration, since the slope (b) is significantly different from zero, the intercept (a) is not significantly different from zero (ISO 8466/1), and the correlation coefficient is higher than 0.999.

Table IV.2 – Linearity of the DPPH method.

Source of variation	**SQ**	**DF**	**MS**	**F calculated**	**Ftab**	
Regression	13773.58784	1	13773.58784	7787.32093	12.83	*
Residual	17.68719687	10	1.768719687			
Pure error	16.34608410	6	2.724347349			
Adjustment error (lack-of-fit)	1.34111277	4	0.335278193	0.12307	12.03	ns
Total variation	13791.27503501	11	13775.356557831			

*- significant difference between F calculated and Ftab ($p<0.05$); ns-difference not significant.

According to the regression analysis, the calculation of the percentage of antioxidant activity, expressed in mM of Trolox (x) was based on the equation 2.

$$x = (y - 1{,}59) / 6{,}19 \qquad \text{Eq. 2}$$

where y = (A initial - A final) / A initial.

4.1.3 Repeatability

The repeatability was calculated from 10 replicates of two quite different wine spirits under constant operating conditions (laboratory, equipment, operator and method) over a short period of time (Monteiro and Bertrand, 1990). The samples were selected according to their total phenolic content: wine spirit aged by the alternative technology with chestnut wood during 12 months (TC1_T12) and wine spirit aged by the traditional technology with a mixture of wood during one month (BM2_T1).

The repeatability was calculated according to the Equation 3.

$$r = 2.26 * \sqrt{2} * SD \qquad \text{Eq. 3}$$

where r-repeatability; SD-standard deviation.

The results obtained, shown in Table IV.3 are satisfactory, with a relative standard deviation of repeatability (RSD) less than 6%. These values reflect the good precision of the method.

Table IV.3 – Repeatability of the DPPH method.

	% inhibition DPPH	
Repetition	**TC1_T12**	**BM2_T1**
r1	74.23	6.12
r2	75.23	6.04
r3	76.11	6.03
r4	69.00	5.52
r5	67.01	5.73
r6	68.66	6.01
r7	64.05	5.72
r8	71.41	5.48
r9	73.26	5.55
r10	65.40	6.30
x	70.44	5.85
SD	4.24	0.29
Repeatability	13.54	0.92
RSD (%)	6.01	4.89

x – mean; SD – standard deviation; RSD – relative standard deviation of repeatability.

The study of the method revealed that it is readily for use in the antioxidant activity analysis of wine spirits from this essay, giving reliable results.

4.2 Comparison of wine spirit's and other food's antioxidant activity

Application of the DPPH method to the wine spirits under study, gave antioxidant activity values between 0.12 and 11.67 mM of Trolox/L. These values were used to carry out a comparison with the antioxidant activity of other foods and beverages reported in the literature – Table IV. 4. It emerged that the food with the greatest antioxidant activity was tea, in particular *Xinyagmajian* green tea (Zhang *et al*., 2013), while food with less antioxidant activity is Sauvignon Blanc (Tauchen *et al*., 2015) wine. The antioxidant activity of the wine spirits under study is lower than the antioxidant activity of oregano essential oil (363 mM/g) (Dutra *et al*., 2019), cherry (32.18-43.05 mM/100mL) (Dragović-Uzelac *et al*., 2007), myrte liqueur (Tuberoso *et al*., 2013) and strawberry tree honey (Tuberoso *et al*., 2013). The wine spirits' antioxidant activity appears to be similar to sugarcane juice (2.3 mg/g) (Ali *et al*., 2019), molasses (1.9 mg/g**)** (Ali *et al*., 2019), some fruits such as strawberries (8.29-11.15 mM/kg) (Dragović-Uzelac *et al*., 2007), juice, white and red wines, nalewkas, beer.

Nalewka is a traditional spirit drink from Poland. It is produced from the maceration and/or infusion of various ingredients in alcohol, and generally has an alcohol strength of 40% but it can even reach 75% in alcohol.

Table IV.4 – Comparison between wine spirit's and other food's antioxidant activity.

Matrix	Antioxidant Activity in Trolox equivalents	Units	Comparison mean WS-F	References
Wine spirits (studied)*	**min: 0.12 mM/L max: 11.67 mM/L**		-	-
Oregano essential oil	363	mM/g	<	**1**
Sugarcane juice	2.3	mg/g	<	**2**
Molasses	1.9	mg/g	<	**2**
Fruits				
Strawberry	8.29-11.15	mM/kg	<	**3**
Sour cherry	32.18-43.05	mM/kg	<	**3**
Blackthorn	24.4-28.15	mM/kg	<	**3**
Cornelian cherry	33.41-39.89	mM/kg	<	**3**
Guava fruits	16.2-32	mM/g	<	**4**
Strawberry tree honey	12	mM/L	<	**5**
Tea	1351-2451	mM/g	<	**6**
Juice:				
Fresh orange juice	81.12; 282.92	mg/100mL mM/100mL	<	**7,8**
Fresh lemon juice	62.54	mg/100mL	>	**7**
Fresh white-grapefruit juice	61.37; 232.37	mg/100mL mM/100mL	>	**7,8**
Fresh tangerine juice	55.28	mg/100mL	>	**7**
Orange juice	381.46	mM/100mL	<	**8**
Apple juice	158.06	mM/100mL	>	**8**
Fresh citron juice	382.78	mM/100mL	<	**8**
Fresh raspberry juice	198.42	mM/100mL	>	**8**
Blackcurrant drink	250.01	mM/100mL	>	**8**
Red grape drink	207.91	mM/100mL	>	**8**
Grape drink	26.43	mM/100mL	>	**8**
Cherry drink	108.27	mM/100mL	>	**8**
Plum drink	78.31	mM/100mL	>	**8**
Apple nectar	188.22	mM/100mL	>	**8**
Blackcurrant nectar	357.59	mM/100mL	<	**8**
Cherry nectar	162.69	mM/100mL	>	**8**
Red Wines	7.9; 139.27-149.21; 1.88-8.59	mM/L; mg/100mL; g/L	<	**5,7,9**
White Wines	8.22; 0.06-2.9; 0.02 - 4,5;	mg/100mL; g/L mM/L;	>; >; ><	**7,9,10,11**
Beer**	38.81 - 795.37	mM/100mL	<>	**12**
Myrte liqueur	26.7	mM/L	<	**5**
Nalewkas**	45-1045	mM/100mL	<>	**13**

1. Dutra *et al*., 2019; **2**. Ali *et al*., 2019; **3**. Dragović-Uzelac *et al*., 2007; **4**. Thaipong *et al*., 2006; **5**. Tuberoso *et al*., 2013; **6**. Zhang *et al*. 2013; **7**. Arnao, 2000; **8**. Bartoszek and Polak, 2016; **9**. Tauchen *et al*., 2015; **10**. Baiano and Varva, 2019; **11**. Serreli *et al*., 2017; **12**. Polak *et al*., 2013; **13**. Polak and Bartoszek, 2015.

* Minimum value - Maximum values ** by EPR spectroscopy method; WS - Wine spirits studied in this work; F - foods; Comparison WS - F: < indicates lower antioxidant activity in the studied wine spirits than in the foods found in the literature; > indicates higher antioxidant activity in the studied wine spirits than in the foods found in the literature; >< indicates higher antioxidant activity in the studied wine spirits than the minimum value of foods found in literature and lower antioxidant activity in the studied wine spirits than the maximum value in the foods found in the literature; <> indicates lower antioxidant activity in the studied wine spirits than the minimum value of foods found in literature and higher antioxidant activity in the studied wine spirits than the maximum value in the foods found in the literature.

These results confirm the reasonable antioxidant activity of the aged wine spirits, which can be seen as contributors to the supply of beneficial compounds to consumer's health, offsetting the negative effect of high alcohol strength, if consumed in moderation. However, it is important to point out that the real effects of the antioxidant activity of the wine spirit should not be so evident than of other foods and drinks (such as fruit juices) because it is consumed in lower quantities.

Analysing the literature, it was realized the presence of many studies on the antioxidant activity of foods, but using different analytical methods, expressing the results in Trolox or in % DPPH inhibition. On the other hand, it is preferable to express the results in % DPPH inhibition to analyse the effects of the ageing technology, kind of wood and ageing time presented below, in order to simplify the work, avoiding falling into the error because the conversion in Trolox is not simple.

4.3 Ageing conditions and antioxidant activity

After one year of ageing, the wine spirits examined showed antioxidant activity values between 2.32 and 73.81% inhibition of DPPH, as shown in Table IV.5 and Table A.1 in Appendix.

Table IV. 5 – Minimum and maximum value of wine spirits antioxidant activity expressed in percentage of DPPH inhibition.

	% inib DPPH
Min	2.32
Max	73.81

The range was very wide probably due to the influence of different factors that determine aged wine spirit's antioxidant activity, as mentioned in 2.3.5. In this case, the factors were the ageing technology (barrels for the traditional technology and stainless steel tanks for the alternative technology), the kind of wood (Limousin, chestnut and mixture of both) and the ageing time. In order to study their effects on the antioxidant activity of the wine spirit, a three-way analysis of variance (ANOVA) was performed.

Through the results obtained by ANOVA, showed in the Table IV.6 is clear that all the factors taken individually are highly significant. However, their interactions were also significant (Te*W, Te*T, W*T), which means that the action of one factor depends on the other one. The interaction between the three factors examined (technology, type of wood and ageing time) was not significant.

Similar results were obtained by Canas *et al*. (2019) for the phenolic compounds of these wine spirits in the first six months of ageing.

Table IV.6 – Three-way ANOVA output.

	SS	**DF**	**MS**	**F**	**p**
Technology	1035.77	1	1035.77	288.906	0,000000***
Wood	3555.63	2	1777.82	495.883	0,000000***
Time	15824.07	4	3956.02	1103.445	0,000000***
Interactions					
Technology*Wood (Te*W)	50.22	2	25.11	7.004	0,002287**
Technology*Time (Te*T)	419.35	4	104.84	29.242	0,000000***
Wood*Time (W*T)	2784.21	8	348.03	97.074	0,000000***
Technology*Wood*Time	51.11	8	6.39	1.782	0,106548
Error	157.75	44	3.59		

** very significant; *** highly significant.
SS – sum of squares; DF – degree of freedom; MS – mean squares; F – Fisher' F; p – level of significance.

4.3.1 Impact of the ageing technology on the wine spirits' antioxidant activity

Comparing the two ageing technologies, there was highly significant difference in the antioxidant activity conferred to the wine spirits (Table IV.7). The wine distillate aged by the alternative technology presented higher antioxidant activity than aged by the traditional one (about 1.5 fold).

It is important to highlight that such significant effect was obtained even with high standard deviation, which reflects the great variability between the barrels and between the tanks. Similar variability was already found for the phenolic composition induced by the traditional technology and by the alternative one (Canas *et al*., 2019).

The results obtained show the alternative technology as a promoter of higher quality wine spirit compared to traditional technology, reinforcing the results of Canas *et al*. (2019).

Table IV.7 – Mean values of antioxidant activity of the wine spirits aged by different technologies.

Ageing technology	%inib DPPH
Traditional (B)	14.49 ± 15.80 a
Alternative (T)	22.15 ± 20.58 b

B – traditional technology; T – alternative technology, means followed by different letters are highly significant different ($p < 0.001$).

4.3.2 Impact of the wood on the wine spirits' antioxidant activity

Anova's results for the effect of different types of wood revealed a highly significant difference between the wine spirits aged with chestnut, Limousin oak and the mixture, despite the high standard deviation – Table IV.8. Such variability can be justified by the antioxidant activity values from both alternative and traditional technology used to calculate the means.

Table IV.8 – Mean values of antioxidant activity of the aged wine spirits according to the kind of wood.

Kind of wood	%inib DPPH
Chestnut (C)	25.91 ± 23.17 c
Limousin (L)	9.05 ± 8.26 a
Mixture (M)	17.53 ± 15.90 b

C – chestnut wood; L – Limousin oak; M – mixture wood, means followed by different letters are highly significant different ($p < 0.001$).

Specifically, the wine spirits aged with chestnut wood exhibited almost 3 times more antioxidant activity than wine spirits aged with Limousin oak, while the mixture of these kinds of wood promoted intermediate mean values. This effect can be ascribed to the richness in phenolic compounds already described by Canas *et al.* (2019), particularly gallic acid, ellagic acid and vanillic acid, which had a strong correlation with the antioxidant activity (Rice-Evans *et al.*, 1996; Zafrilla *et al*., 2001). The outcomes confirm those of Canas *et al.* (2008a) and Ziyatdinova *et al*. (2014) obtained for the traditional technology.

Alañón *et al*. (2011a) also showed in their work about wines, how the use of the different kinds of wood affected the phenolic composition, due to the different wood components.

4.3.3 Impact of the ageing time on the wine spirits' antioxidant activity

Table IV.9 shows that the wine spirits' antioxidant activity increased continuously over the time, at least throughout the first year of ageing, to which the analyses refer. From eight to 30 days of ageing the antioxidant activity doubles, from one month to six months it triples, then from six months to one year it increases more than 1.5 times.

These results seem to be related to the release of the wood compounds, which is more pronounced in the first months of ageing (Canas *et al*., 2002); such evolution is likely due to a greater concentration gradient of phenolic compounds between the wood and the distillate, because the wine distillate is characterised by high ethanol content with many volatile compounds but is devoid of phenolic compounds other than volatile phenols (Caldeira *et al*., 2016).

Table IV.9 – Mean values of antioxidant activity of the aged wine spirits according to the ageing time.

Ageing time	%inib DPPH
8 days	4.06 ± 2.03 a
15 days	5.64 ± 2.72 b
30 days	8.65 ± 4.56 c
180 days	26.25 ± 12.93 d
365 days	41.97 ± 19.17 e

Means followed by different letters are highly significant different ($p < 0.001$).

In the first year, the percentage of DPPH inhibition increased at about ten fold. This increment is in accordance with the previous study, made by Canas *et al*. (2008a) which showed the highly significant increment of antioxidant activity of phenolic acids fraction during the ageing period. Pecić

et al. (2012) and Da Porto *et al*. (2000) also observed the increase of phenolic composition and antioxidant activity over the ageing time.

The standard deviation's values were high, as for the ageing technology and the kind of wood.

4.3.4 Study of the interactions between the ageing factors

4.3.4.1 Interaction between the ageing technology and the kind of wood

The results shown in Figure 4.2 and Table A.2 (in Appendix), demonstrate that the alternative technology with chestnut wood confers greater antioxidant activity than the other modalities. Comparing the alternative technology with chestnut wood and the alternative technology with Limousin oak, the first one induced 2.5 more antioxidant activity, while the mixture promoted an antioxidant activity between the chestnut and the Limousin oak. The results are similar for the traditional technology but with lower value of antioxidant activity for the corresponding kind of wood. The antioxidant activity of the wine spirit aged in the traditional technology with the chestnut wood (22.24%) was similar to the antioxidant activity of the wine spirit aged by the alternative technology with mixture of wood (23.04%).

For the traditional technology, the difference of antioxidant activity between wine spirits aged with chestnut and with mixture was 8.02%, while the difference between the wine spirits aged with mixture and Limousin oak was 7.21%. Instead, for the alternative technology, the difference in antioxidant activity between the wine spirit aged with chestnut and that with mixture was 8.37%, while between that aged in mixture and Limousin was 10.94%. Therefore, the results were similar for both technologies regard to the kind of wood, as the wine spirit aged with wood mixture had an antioxidant activity closer to chestnut wood rather than Limousin oak.

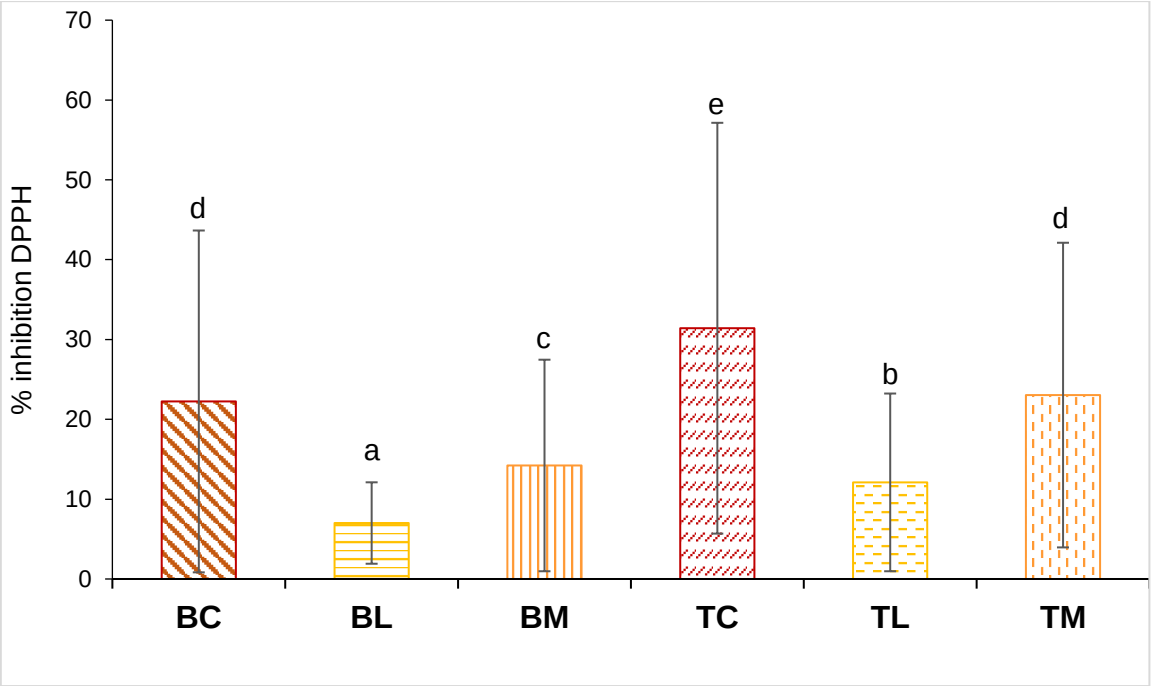

Figure 4.2 – Percentage of inhibition of DPPH induced by the different ageing technologies with the different kinds of wood; BC – chestnut barrels BL – Limousin oak barrels; BM – woods mixture barrels; TC – stainless steel tanks with chestnut wood staves; TL – stainless steel tanks with Limousin oak staves; TM – stainless steel tanks with woods mixture staves. Different letters in the bars indicate a very significant difference ($p<0.01$).

The significant interaction is due to the quantitative differences in the antioxidant activity between technologies for each kind of wood.

Moreover, as already studied by Canas *et al*. (2019), the use of micro-oxygenation technique in the alternative technology has given rise to greater differences in the phenolic composition between aged wine spirits in chestnut wood and those aged in Limousin oak, so the micro-oxygenation seem to facilitate the ageing process on account of the extraction and oxidation phenomena developed in the alternative technology.

The study of the interaction between the kind of wood and the technology showed that with the alternative technology and the chestnut wood promoted higher nutraceutical quality. This result seems to be correlated with the faster enrichment in wood derived compounds in stainless steel tanks.

These results were expected taking into account the higher concentration of phenolic compounds and the higher porosity of chestnut wood, which favours their release, already observed in the traditional ageing research works (Canas, 2017).

4.3.4.2 Interaction between the ageing technology and the ageing time

Regarding the interaction between the ageing technology and the ageing time factors - Figure 4.3 and Table A.3 (in Appenidix), the two kinetics representing the wine spirits aged by the technologies that had an ever increase trend over the first year of ageing.

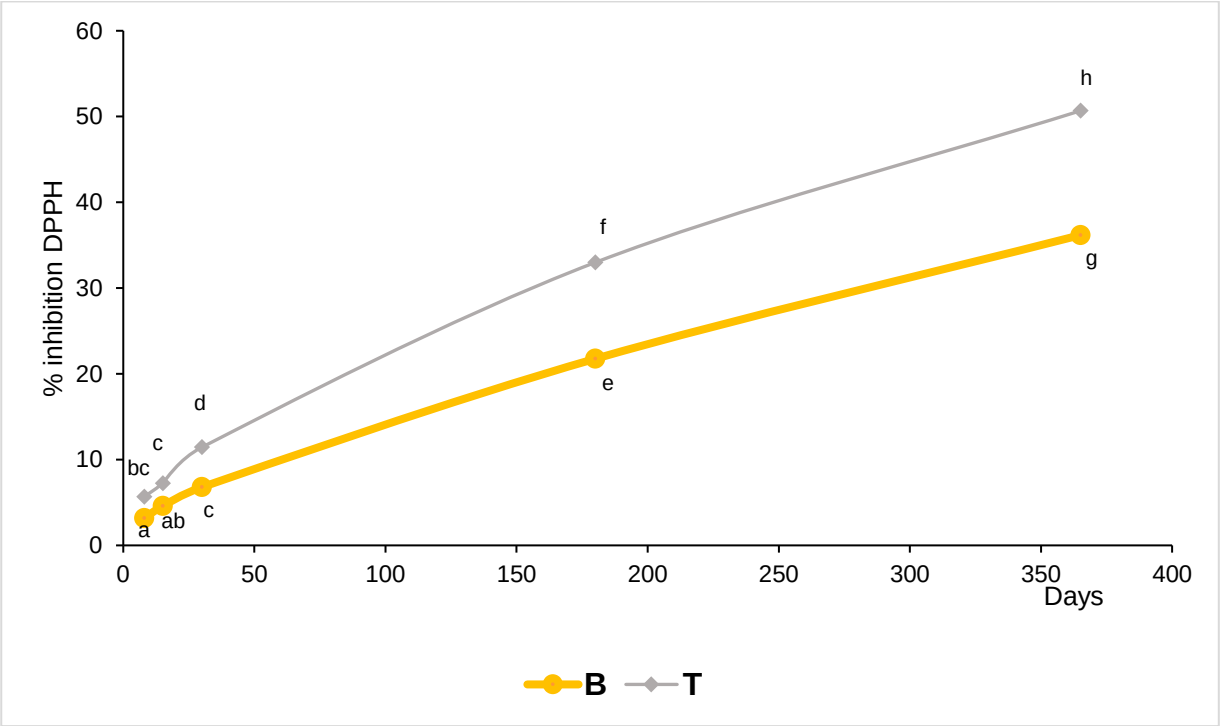

Figure 4.3 – Variation of percentage of DPPH inhibition induced by the different ageing technologies over the ageing time. B – traditional technology; T – alternative technology; different letters indicate highly significant difference ($p < 0.01$).

Analysing in detail the different time intervals it is possible to notice how in the period between 8 and 15 days the antioxidant activity increased by 1.4 fold in wine spirits aged by the traditional technology, while 1.3 fold in those aged by the alternative technology. In the period between 15 and 30 days it increased 1.5 fold for the traditional technology and 1.6 fold for the alternative technology. From one to six months it increased by 3.2 times for the traditional technology, 2.9 times for the alternative technology. Between six months and one year it increased 1.7 fold in the traditional technology and 1.5 fold for the alternative technology. The results, made evident by the Figure 4.3, showed that the both curves were continuously increasing. The curve relative to the alternative technology showed an antioxidant activity highly than the traditional technology, although both reflected the increase in antioxidant activity over the time.

Splitting the antioxidant activity in the different months, it seems to increase more in the first month of ageing with an increase of twice at the end of the first month, comparing it with the first eight days. It seems that the increase after the first six months decreases, probably due to the fact that in the initial period of ageing there is a greater concentration gradient of the phenolic compounds between the wood and the wine spirit (Canas *et al*., 2002).

Concerning the analysis of the combination of the effects of different technology over time, highlighted a highly increase between one month and six months. And, confirming what was written above, it seems that the concentration gradient of the compounds present in the wood and wine distillate greatly influences the antioxidant activity of aged wine spirits.

4.3.4.3 Interaction between the kind of wood and the ageing time

Figure 4.4 and Table A.4 (in Appendix) report the evolution of wine spirits' antioxidant activity associated with the different kinds of wood over the time. The results revealed higher antioxidant activity in the wine spirits aged with chestnut and lower in those aged with Limousin oak over the entire period.

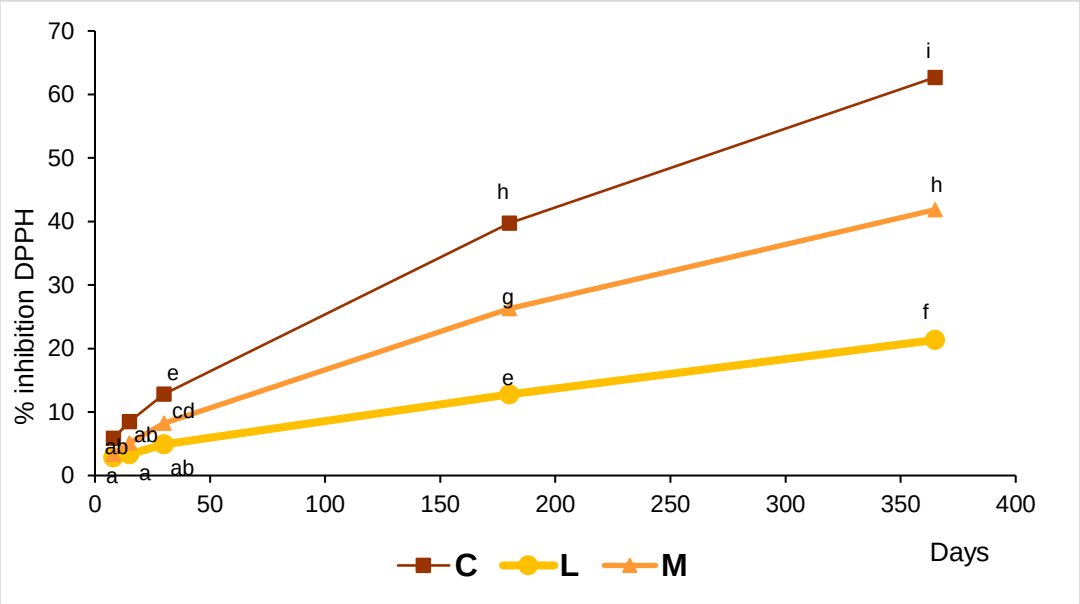

Figure 4.4 – Variation of percentage of DPPH inhibition associated with different kinds of wood over the ageing time. C – chestnut wood; L – Limousin oak; M – mixture of wood; different letters indicate highly significant difference ($p < 0.01$).

In addition, the results showed that the evolution pattern of antioxidant activity depends on the wood used, justifying the significant interaction observed:

- Between 8 and 15 days it increased 1.5 fold for the chestnut wood, 1.2 fold for the Limousin oak, and 1.5 fold for the mixture, which suggest the stronger influence of the chestnut wood within the mixture.

- Between 15 and 30 days it increased 1.5 fold the wine spirit aged in chestnut and also in Limousin oak, and 1.6 fold the wine spirit aged in the mixture (likely resulting from a synergistic effect of the two kinds of wood).

- From one month to six months the antioxidant activity increased 3.1 fold in the wine spirit aged with chestnut wood, 2.6 fold the wine spirit aged with Limousin oak and 3.2 fold the wine spirit aged with the wood mixture (indicating that the synergistic effect prevailed).

- From six months to one year, the antioxidant activity of chestnut aged wine spirits increased by 1.6 fold, 1.7 fold for the wine spirit aged with Limousin oak and 1.6 fold in the wine spirit aged with the mixture of the two different kinds of wood, as in the 15 first days of ageing.

4.4 Correlation analysis between the antioxidant activity and the total phenolic content

Taking into account the literature about the correlation between the percentage of DPPH inhibition (AA %) and the total phenolic content (TPC) of the wine spirits (Da Porto *et al.*, 2000; Canas *et al.*, 2008a), it was decided to study the correlation between these two variables for the wine spirits of this project.

The results confirm that a positive and significant correlation between the antioxidant activity and the total phenolic content exists (Table IV.10, Figure 4.5).

Table IV.10 - Output of the correlation analysis between antioxidant activity (AA) and total phenolic content (TPC)

	AA(%)	**TPC**
AA (%)	1	
TPC	0.95921**	1

AA (%) - expressed in percentage;
TPC – Total Phenolic Content;
** significant correlation ($p<0.01$)

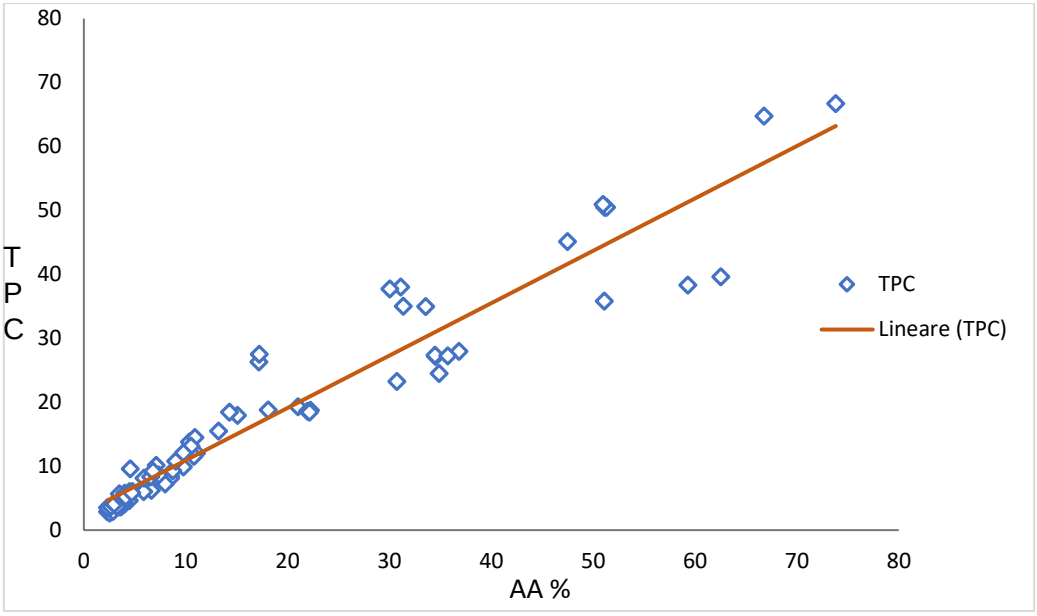

Figure 4.5 – Correlation analysis between the antioxidant activity and the total phenolic content.

This finding allows to state that all the factors that determined the presence of the phenolic compounds in the wine spirit were conclusive for the antioxidant activity. For this reason, it is important to search, among the different technologies and among the different kind of wood, the best combination to reach our goal; that is, to produce quality wine spirits (such as those obtained through traditional technology) in the shortest time, in the most sustainable way. Not only with regard to wine spirits, but for example the study made by Aljadi and Kamaruddin (2004) showed that most of the phenolic compounds extracted from honey possess antioxidant activity.

4.5 Correlation analysis between the antioxidant activity and the low molecular weight phenolic compounds.

To further examine the relationship between the antioxidant activity and the phenolic composition of the aged wine spirits, another correlation analysis was performed. The phenolic composition of wine spirit was previously determined by HPLC for the first six months of ageing.
Correlations were calculated taking into account both ageing technologies and each technology separately (Table IV. 11).

The results showed a positive and very significant correlation between antioxidant activity and all the parameters analysed, apart from the scopoletin which was only significant.

Among the different compounds, the highest correlation was found between ellagic acid and antioxidant activity, which was in agreement with the findings of Priyadarsini *et al*. (2002).

Table IV.11 - Correlation coefficients between the antioxidant activity (AA) and the phenolic composition of all aged wine spirits

	Both technologies	Traditional (B)	Alternative (T)
	AA	**AA**	**AA**
TPC	0,96155**	0,98123**	0,97589**
LMWC	0,97381**	0,99281**	0,95838**
gall	0,87400**	0,96520**	0,92606**
van	0,87841**	0,81072**	0,93435**
syrg	0,81126**	0,89040**	0,90661**
ellag	0,96995**	0,97379**	0,97378**
vanil	0,89382**	0,91627**	0,92397**
syrde	0,84844**	0,86304**	0,86092**
cofde	0,81660**	0,87963**	0,77489**
sipde	0,81748**	0,88480**	0,80872**
umb	0,84942**	0,85488**	0,88125**
scop	0,32321*	0,37458*	0,42102*

n=59 (both), 36 (traditional), 23 (alternative); * significant correlation ($p<0.05$); ** significant correlation ($p<0.01$); LMWC – sum of low molecular weight phenolic compounds quantified by HPLC; TPC – total phenolic content; gall – gallic acid; van – vanillic acid; syrg – syringic acid; ellag – ellagic acid; vanil – vanillin; syrde – syringaldehyde; cofde – coniferaldehyde; sipde – sinapaldehyde; umb – umbelliferone; scop – scopoletin.

Considering both technologies, the correlations between the antioxidant activity and vanillin, vanillic acid and gallic acid also stood out, followed by the correlations between antioxidant activity and the other phenolic aldehydes. The study made by Sroka and Cisowski (2003) also showed the strong gallic acid's antioxidant activity. Ibrahim *et al.* (2012) reported the antioxidant activity of syringaldehyde.

Specifically, the wine spirits aged in barrels had slightly higher correlations between antioxidant activity and gallic acid, ellagic acid, syringaldehyde, coniferaldehyde and sinapaldehyde.

Regarding the alternative technology, slightly higher correlations were found between antioxidant activity and vanillic acid, syringic acid and vanillin.

The partial outcomes of the correlation analysis (using only wine spirits from wooden barrels or wine spirits from the stainless steel tanks with staves and micro-oxygenation) suggest that the relationship

between the antioxidant activity and the low molecular weight phenolic compounds is similar in both ageing technologies.

4.6 Principal Components Analysis

The Multivariate analysis was performed to make a global analysis of the data; that is, how the various factors examined (ageing technology, type of wood and ageing time) influenced all together the quality of the wine spirit taking into account the antioxidant activity, the total phenolic content and the low molecular weight phenolic compounds concentrations determined by HPLC.

Firstly, the projection of the wine spirits sampled over the ageing time (Figure 4.6) give a general picture of the effects of ageing. The time and the ageing technology are mainly responsible for the greatest distinction between wine spirits. The two principal components explain 93.91% of the total variance.

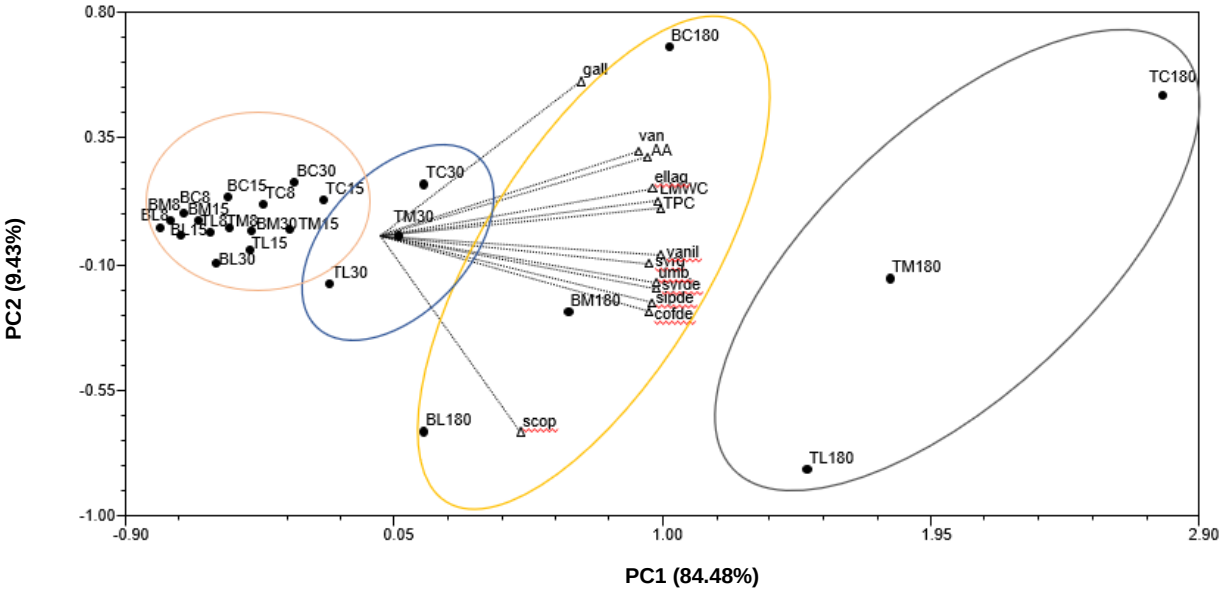

Figure 4.6 – Projection of the aged wine spirits, antioxidant activity, phenolic composition and low molecular weight compounds in the space defined by the two first principal components.

Wine spirits identification: ageing technology (B – traditional technology; T – alternative technology); kind of wood (L – Limousin oak; C – chestnut; M – mixture of both woods); sampling time (8 – 8 days; 15 – 15 days; 30 – 30 days; 180 – 180 days). AA – antioxidant activity; LMWC – sum of low molecular weight phenolic compounds quantified by HPLC; TPI – total phenolic index; gall – gallic acid; van – vanillic acid; syrg – syringic acid; ellag – ellagic acid; vanil – vanillin; syrde – syringaldehyde; cofde – coniferaldehyde; sipde – sinapaldehyde; umb – umbelliferone; scop – scopoletin.

Analysing the different groups, it turned out the presence of a substantial difference between the two ageing technologies.

The first principal component (PC1), which explains 84.48% of the total variance, makes the splitting of the wine spirits based on ageing time. It is possible to observe that the 180-days-aged wine spirits by alternative technology are all on the right side of the plan, while the 180-days-aged wine spirits using traditional technology are in the central part.

Wine spirits aged 30 days using alternative technology are slightly to the left. In the first period of ageing there was no noticeable difference between the different wine spirits. As already mentioned (*vide* 4.3.3), during the first month (samplings at 8 and 15 days) there was not a clear distinction between the two technologies, probably because the extraction of the wood compounds by the distillate was intense, while after the first 30 days there was an evolution of the compounds present, increasing and determining the complexity of the wine spirit.

The results show that PC1 has strong positive loading vectors for antioxidant activity, total phenolic content, LMWC, vanillin, syringaldehyde, umbelliferone, ellagic acid, sinapaldehyde, coniferaldehyde, syringic acid.

The second principal component (PC2), which explains 9.43% of the total variance, makes the separation according to the kind of wood. The wine spirits aged with Limousin wood are located in the lower part of the plan, as is the scopoletin; probably because this compound is a chemical marker of Limousin oak (Canas *et al*., 2019). Unlike the wine spirits aged with chestnut wood, which are located at the top of the plan, along with gallic acid, which is chemical marker of this kind of wood (Canas *et al*., 2019), and vanillic acid. The wine spirits aged with the wood mixture are mainly located at the middle part of the plain as a result of their intermediate characteristics.

It is important to stress the strong positive loading vector of antioxidant activity for PC2, which confirms the close relationship with chestnut wood; that is, the chestnut wood favoured greater evolution of the wine spirit in terms of antioxidant activity due to its higher phenolic content.

Subsequently, from the results, it was decided to apply the multivariate analysis to the results obtained at 180 days, in order to better understand the relationship between the wine spirits' features and the factors under analysis. The results of this second analysis are presented below.

From the phenogram (Figure 4.7) it is evident the differentiation between two groups: one consisting of aged wine spirits using the traditional technology; the other consisting of wine spirits aged by the alternative technology.

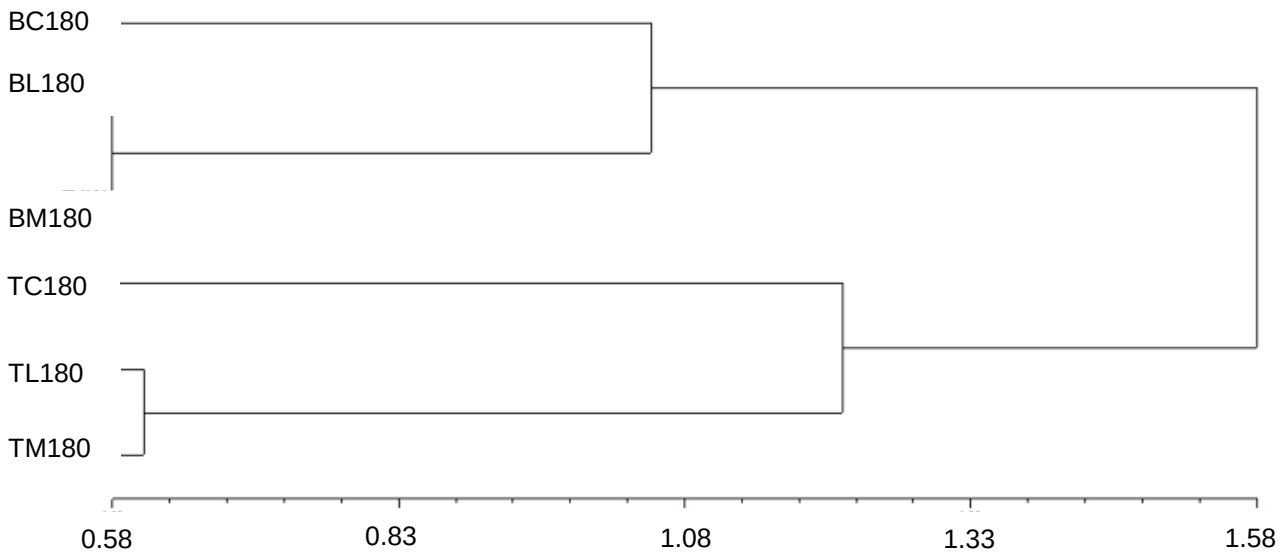

Figure 4.7 – Phenogram of UPGMA clustering analysis of 180 days-aged wine spirits by the traditional and alternative technologies according to antioxidant activity, total phenolic composition and low molecular weight compounds determined by HPLC. Ageing technology (B – traditional technology; T – alternative technology); kind of wood (L – Limousin oak; C – chestnut; M – mixture of both woods); sampling time 180 days.

Therefore, through the analysis of the PCA (Figure 4.8), whose two principal components PC1 and PC2 explain 96.74% of the total variance, it was evident that the first component (PC1), accounting for 75.15% of the total variance, splitting the wine spirits according to the ageing technology. The wine spirits aged by the alternative technology are located at positive values of this components, while those aged by the traditional technology are placed at the negative values.

PC1 has strong positive loading vectors for antioxidant activity, TPC, LMWC, vanillin, vanillic acid, syringic acid, umbelliferone, ellagic acid, syringaldehyde and sinapaldehyde.

Furthermore, PC2, which explains 21.59% of the total variance, separate the wine spirits according to the kind of wood. The wine spirits aged with Limousin oak are in the negative part of the axis, while those aged with chestnut wood are in the positive part. The wine spirits aged with both kinds of wood are in the central part.

The main negative loading vector is scopoletin, and the main positive loading one is gallic acid.

These results reinforce the role of scopoletin and gallic acid as chemical markers of Limousin oak and chestnut, respectively, regardless of the ageing technology. Indeed, they are in accordance with those of Canas *et al*. (2019), which stated that the high concentration of gallic acid and scopoletin are key elements that make the distinction of chestnut from Limousin oak.

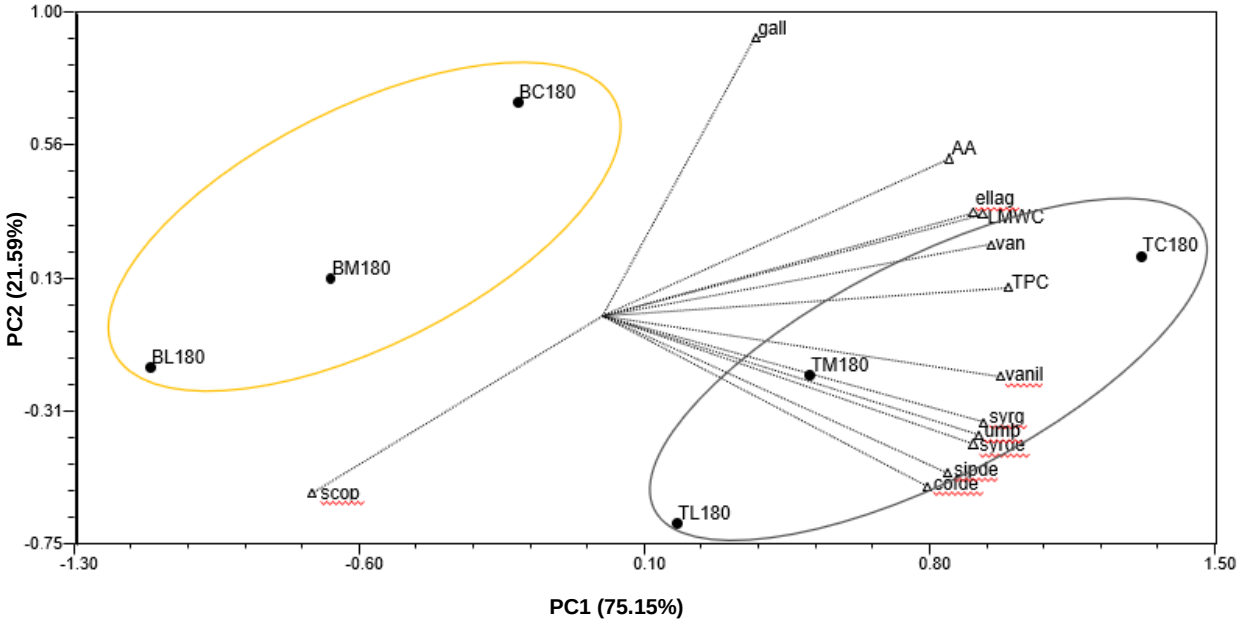

Figure 4.8 – Projection of the six-month aged wine spirits, antioxidant activity, phenolic composition and low molecular weight compounds in the space defined by the two first principal components. Ageing technology (B – traditional technology; T – alternative technology); kind of wood (L – Limousin oak; C – chestnut; M – mixture of both woods); AA – antioxidant activity; LMWC – sum of low molecular weight phenolic compounds quantified by HPLC; TPI – total phenolic index; gall – gallic acid; van – vanillic acid; syrg – syringic acid; ellag – ellagic acid; vanil – vanillin; syrde – syringaldehyde; cofde – coniferaldehyde; sipde – sinapaldehyde; umb – umbelliferone; scop – scopoletin.

The global analysis of the data at six months of ageing corroborated the results of ANOVA and of the correlation analysis, emphasizing the relationship between the antioxidant activity of the wine spirits and their phenolic composition as well as the pivotal role of the ageing technology and the kind of wood on these qualitative features.

The alternative technology using staves and micro-oxygenation, especially in the presence of chestnut wood, seems to be a very promising option to enhance the wine spirits' antioxidant activity.

5. CONCLUSIONS

Considering the experimental conditions of this study, it is possible to conclude:

- The analytical method for the determination of the antioxidant activity of wine spirits based on DPPH was successfully adapted. Moreover, the study of the method showed its good performance with regard to practicability, linearity and repeatability. Therefore, it was readily for use in the antioxidant activity analysis of wine spirits from this essay, giving reliable results in a short analysis time. The analysis of 75 samples proved the real applicability of the method to distinguish wine spirits according to the ageing technology, the type of wood used and the ageing time. This method can also be used in other scientific works and in routine analysis.

- Application of the DPPH method to the wine spirits under study, gave antioxidant activity values between 0.12 and 11.67 mM of Trolox/L. Comparison with the antioxidant activity of other foods and beverages reported in the literature evidenced the reasonable antioxidant activity of this spirit drink, whether obtained by the traditional technology or by the alternative one. Hence, it can be seen as a contributor to the supply of beneficial compounds to consumer's health, offsetting the negative effect of high alcohol strength, if consumed in moderation. However, it is important to point out that the real effects of the antioxidant activity of the wine spirit should not be so evident than of other foods and drinks (such as fruit juices) because it is consumed in lower quantities.

- The ageing technology (traditional or alternative), the type of wood used (Limousin oak, chestnut or the mixture of these two types) and the ageing time are determining factors of the wine spirits' antioxidant activity.

- It is noteworthy how the wine spirits aged through alternative technology (in stainless steel tanks with staves and micro-oxygenation) acquire higher antioxidant activity (higher percentage of DPPH inhibition) as a consequence of a greater enrichment in phenolic compounds than through the traditional technology (in wooden barrels), for the same ageing time.

- Regarding the contribution of wood, the chestnut promoted greater antioxidant activity than the Limousin oak, which was correlated with higher contents of phenolic compounds in the corresponding wine spirit, mainly through the alternative technology. Among these compounds, ellagic acid exhibited the strongest positive correlation with the antioxidant

activity, followed by other phenolic acids (gallic, vanillic and syringic acids) and phenolic aldehydes.

- The antioxidant activity increased continuously over the time in the first 12 months of ageing but more markedly in the wine spirits produced by the alternative technology combined with chestnut wood; that is, these ageing conditions allowed enhancing the nutraceutical quality, adding more value to the spirit drink, along with a faster and less expensive ageing process.

- Without a doubt, as demonstrated by this work, the alternative technology leads to a high quality wine spirit, but the link with tradition is still important. Taking into account the current events of our planet, attention to sustainability, we could think of producing wine spirits aged with alternative technology to produce cheaper products more sustainable with less environmental damage, and limit the production of traditional products for the consumer that requires in a wine spirit the art of the traditional method of ageing the distillate, that is, in wooden barrel.

6. FUTURE PERSPECTIVES

Further work should be carried out using different methods available for the antioxidant activity analysis, such as ABTS radical scavenging capacity, Oxygen Radical Absorbance Capacity (ORAC) and Ferric Reducing Antioxidant Power (FRAP), to better characterize this feature of the wine spirits. More detailed experiments are also required to fully elucidate the role of individual phenolic compounds, namely phenolic aldehydes and tannins, in the aged wine spirits' antioxidant activity.

Research on the *in vivo* antioxidant activity of wine spirit is also required for a thorough understanding of this topic.

It would also be interesting to evaluate the influence of other kinds of wood, such as *Quercus pyrenaica* and *Quercus faginea* in the wine spirits antioxidant activity and phenolic composition according to the ageing technology, in order to understand the potential of using other kinds of wood, and to evaluate the best choices in the wine spirit production process.

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APPENDIX

Table A.1 – Antioxidant activity of wine spirits under study.

Technology	Wood	Time	AA (% inhibitionDPPH)
B	C	8d	4,474
B	C	8d	3,983
B	C	8d	3,731
B	L	8d	2,333
B	L	8d	2,533
B	L	8d	2,842
B	M	8d	2,322
B	M	8d	3,655
B	M	8d	2,655
T	C	8d	8,501
T	C	8d	8,513
T	L	8d	3,455
T	L	8d	3,110
T	M	8d	4,771
B	C	15d	7,968
B	C	15d	6,616
B	C	15d	5,859
B	L	15d	2,971
B	L	15d	3,250
B	L	15d	2,992
B	M	15d	3,593
B	M	15d	4,202
B	M	15d	3,669
T	C	15d	11,050
T	C	15d	10,813
T	L	15d	3,493
T	L	15d	4,031
T	M	15d	6,997
T	M	15d	7,113
B	C	30d	9,475
B	C	30d	9,770
B	C	30d	8,720
B	L	30d	4,499
B	L	30d	4,101
B	L	30d	4,708
B	M	30d	5,894
B	M	30d	7,355
B	M	30d	6,554
T	C	30d	21,018
T	C	30d	15,074
T	L	30d	6,781

T	L	30d	4,555
T	M	30d	10,356
T	M	30d	10,895
B	C	180d	34,530
B	C	180d	34,881
B	C	180d	30,739
B	L	180d	10,537
B	L	180d	9,022
B	L	180d	9,768
B	M	180d	21,975
B	M	180d	22,233
B	M	180d	22,139
T	C	180d	51,122
T	C	180d	47,462
T	L	180d	17,195
T	L	180d	17,228
T	M	180d	33,565
T	M	180d	31,370

B – traditional technology; T – alternative technology; C – chestnut wood; L – Limousin oak; M – mixture wood; d – day.

Table A.2 Percentage of DPPH inhibition of the interaction between ageing technology and kind of wood.

Technology-Wood	%inib DPPH
BC	22.24 ± 21.41
BL	7.01 ± 5.10
BM	14.22 ± 13.25
TC	31.41 ± 25.73
TL	12.10 ± 11.13
TM	23.04 ± 19.08

B – traditional technology; T – alternative technology; C – chestnut wood; L – Limousin oak; M – mixture wood.

Table A.3 Percentage of DPPH inhibition of the interaction between technology and ageing time.

	% inib DPPH	
Ageing time	**Traditional (B)**	**Alternative (T)**
8 days	3.17 ± 0.80 a	5.67 ± 2.67 bc
15 days	4.57 ± 1.81 ab	7.25 ± 3.22 c
30 days	6.79 ± 2.17 c	11.45 ± 5.93 d
180 days	21.76 ± 10.30 e	32.99 ± 14.42 f
365 days	36.17 ± 18.66 g	50.67 ± 17.91 h

Means followed by different letters are highly significant different ($p < 0.001$).

Table A.4 Percentage of DPPH inhibition of the interaction between kind of wood and ageing time.

	% inibit DPPH		
Days	**Chestnut (C)**	**Limousin (L)**	**Mixture (M)**
8	5.84 ± 2.45 bc	2.85 ± 0.45 a	3.35 ± 1.10 ab
15	8.46 ± 2.38 d	3.35 ± 0.44 a	5.11 ± 1.79 ab
30	12.81 ± 5.24 e	4.93 ± 1.06 ab	8.21 ± 2.27 cd
180	39.75 ± 8.96 h	12.75 ± 4.11 e	26.26 ± 5.72 g
365	62.69 ± 8.46 i	21.35 ± 8.62 f	41.86 ± 8.51 h

Means followed by different letters are highly significant different ($p < 0.001$).

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STATEMENT

The student Anna Nocera made the minor corrections in her Master Dissertation entitled "**Impact of an alternative ageing technology using micro-oxygenation on the wine spirit's antioxidant activity**" requested by the jury of the Master defense that took place on October 31, 2019.

The final version of the Master Dissertation meets the required conditions to be delivered at the University of Lisbon.

Dois Portos, November 6, 2019

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